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(54) Title: SYNTHETIC MAMMALIAN CHROMOSOME AND METHODS FOR CONSTRUCTION (57) Abstract <p>The invention relates to the field of gene therapy, gene expression, and vectors for these uses. In particular, the invention relates to a method for producing structurally intact large repeating units of DNA, especially useful for the stable cloning of alpha satellite DNA, and to the development and use of a synthetic or artificial chromosome for gene expression and gene therapy in mammals, and especially humans. The invention allows the controlled construction of stable synthetic or artificial chromosomes constructed from isolated segments of purified DNA. Functional minimal segments preferably include centromeric DNA, telomeric DNA, and genomic DNA. The artificial chromosome performs the essential chromosomal functions of naturally-occurring chromosomes so as to permit the chromosome to function as an effective vector for gene therapy.</p>		

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Synthetic Mammalian Chromosome and Methods for Construction

Statement as to Rights to Inventions Made Under Federally-Sponsored Research and Development

5 Part of the work performed during development of this invention utilized
U.S. Government funds. The U.S. Government has certain rights in this
invention.

Field of the Invention

10 The invention relates to the field of gene expression and gene therapy,
and to novel vectors for these uses. In particular, the invention relates to the
development and use of a synthetic or artificial chromosome as a vector for gene
expression and gene therapy, especially in humans. The invention enables the
controlled construction of stable synthetic or artificial chromosomes from isolated
purified DNA. With this DNA, a functional chromosome is formed in a cell and
15 maintained as an extrachromosomal element. The artificial chromosome
performs the essential chromosomal functions of naturally-occurring
chromosomes so as to permit the chromosome to function as an effective vector
for gene therapy when therapeutic DNA is included in the chromosome.

Background of the Invention

20 The genetic manipulation of cells aimed at correcting inherited or
acquired disease is referred to as gene therapy. Until now, most clinical studies
in this field have focused on the use of viral gene therapy vectors. Based on the
results of these studies, it is becoming clear that current viral gene therapy vectors
have severe clinical limitations. These include immunogenicity, cytopathicity,
25 inconsistent gene expression, and limitations on the size of the therapeutic gene.

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For these reasons, much attention has been recently focused on the use of non-viral gene therapy vectors.

In particular, synthetic mammalian chromosomes would be useful vectors for facilitating a variety of genetic manipulations to living cells. The advantages of synthetic mammalian chromosomes include high mitotic stability, consistent
5 and regulated gene expression, high cloning capacity, and non-immunogenicity.

Artificial chromosomes were first constructed in *S. cerevisiae* in 1983 (Murray *et al.*, *Nature* 305:189-193 (1983), and in *S. pombe* in 1989 (Hahnenberger *et al.*, *Proc. Natl. Acad. Sci. USA* 86:577-581 (1989). For many
10 reasons, however, it has not been obvious whether similar vectors could be made in mammalian cells.

First, multicellular organisms (and thus the progenitors of mammalian cells) diverged from yeast over 1 billion years ago. Although there are similarities among living organisms, in general, the similarities among two
15 organisms are inversely related to the extent of their evolutionary divergence. Clearly, yeast, a unicellular organism, is radically different biologically from a complex multicellular vertebrate.

Second, yeast chromosomes are several orders of magnitude smaller than mammalian chromosomes. In *S. cerevisiae* and *S. pombe*, the chromosomes are
20 0.2 to 2 megabases and 3.5-5.5 megabases in length, respectively. In contrast, mammalian chromosomes range in size from approximately 50 megabases to 250 megabases. Since there is a significant difference in size, it is not clear, *a priori*, whether constructs comparable to yeast artificial chromosomes can be constructed and transfected into mammalian cells.

Third, yeast chromosomes are less condensed than mammalian chromosomes. This implies that mammalian chromosomes rely on more complex chromatin interactions in order to achieve this higher level of structure. The
25 complex structure (both DNA structure and higher order chromatin structure) of mammalian chromosomes calls into question whether artificial chromosomes can be created in mammalian cells.
30

Fourth, yeast centromeres are far less complex than mammalian centromeres. In *S. cerevisiae*, for example, the centromere is made up of a 125 bp sequence. In *S. pombe*, the centromere consists of approximately 2 to 3 copies of a 14 kb sequence element and an inverted repeat separated by a core region (~7 kb). In contrast, human centromeres are made up of several hundred kilobases to several megabases of highly repetitive alpha satellite DNA. Furthermore, in mammalian centromeres, there is no evidence for a central core region or inverted repeats such as those found in *S. pombe*. Thus, unlike yeast centromeres, mammalian centromeres are extremely large and repetitive.

Fifth, yeast centromeres have far fewer spindle attachments than mammalian centromeres (Bloom, *Cell* 73:621-624 (1993)). *S. cerevisiae*, for example, has a single microtubule attached to the centromere. In *S. Pombe*, there are 2-4 microtubules attached per centromere. In humans, on the other hand, there are several dozen microtubules attached to the centromere of each chromosome (Bloom, *Cell* 73:621-624 (1993)). This further illustrates the complexity of mammalian centromeres compared to yeast centromeres.

Together, these differences are significant, and do not suggest that a result in yeast can be reasonably expected to be transferable to mammals.

Normal mammalian chromosomes are comprised of a continuous linear strand of DNA ranging in size from approximately 50 to 250 megabases. In order for these genetic units to be faithfully replicated and segregated at each cell division, it is believed that they must contain at least three types of functional elements: telomeres, origins of replication, and centromeres.

Telomeres in mammals are composed of the repeating sequence (TTAGGG)_n and are thought to be necessary for replication and stabilization of the chromosome ends. Origins of replication are necessary for the efficient and controlled replication of the chromosome DNA during S phase of the cell cycle. Although mammalian origins of replication have not been well-characterized at the sequence level, it is believed that they are relatively abundant in mammalian DNA. Finally, centromeres are necessary for the segregation of individual

chromatids to the two daughter cells during mitosis to ensure that each daughter cell receives one, and only one, copy of each chromosome. Like origins of replication, centromeres have not been defined at the sequence level. Alpha satellite DNA may be an important centromeric component (Haaf *et al.*, *Cell* 70:681-696 (1992); Larin *et al.*, *Hum. Mol. Genet.* 3:689-695 (1994); Willard, *Trends in Genet.* 6:410-415 (1990)). But there are cases of mitotically stable abnormal chromosome derivatives that apparently lack alpha satellite DNA (Callen *et al.*, *Am. J. Med. Genet.* 43:709-715 (1992); Crolla *et al.*, *J. Med. Genet.* 29:699-703 (1992); Voullaire *et al.*, *Am. J. Hum. Genet.* 52:1153-1163 (1993); Blennow *et al.*, *Am. J. Hum. Genet.* 54:877-853 (1994); Ohashi *et al.*, *Am. J. Hum. Genet.* 55:1202-1208 (1994)). Thus, at this time, the composition of the mammalian centromere remains poorly understood.

While others have claimed to have produced "artificial" chromosomes in mammalian cells, no one has ever produced an artificial chromosome that contains only exogenous DNA. In each of these previous cases, the investigators either modified an existing chromosome to make it smaller (the "pare-down" approach) or they integrated exogenous DNA into an existing chromosome which then broke to produce a chromosome fragment containing endogenous sequences from the preexisting chromosome (the "fragmentation" approach). In the present invention, exogenous DNA sequences are introduced into human cells and form stable synthetic chromosomes without integration into endogenous chromosomes.

Among the pare-down approaches, three specific strategies have been used: (1) telomere directed truncation via illegitimate recombination (Barnett, M.A. *et al.*, *Nucleic Acids Res.* 21:27-36 (1993); Farr, C.J. *et al.*, *EMBO J.* 14:5444-54 (1995)) (2) alpha satellite targeted telomere insertion/truncation via homologous recombination (Brown, K.E. *et al.*, *Hum Mol. Genet.* 3:1227-37 (1994)) (3) formation/breakage of dicentric chromosomes (Hadlaczky, G., *Mammalian Artificial Chromosomes*, U.S. Patent 5,288,625 (1994)).

Barnett *et al.* (*Nucleic Acids Res.* 21:27-36 (1993)), Farr *et al.* (*EMBO J.* 14:5444-54 (1995)), and Brown *et al.* (*Hum Mol. Genet.* 3:1227-37 (1994))

describe methods for fragmenting endogenous chromosomes by transfecting telomeric DNA and a selectable marker into mammalian cells. In each case, a truncated chromosome was created that was smaller than the original chromosome. The resulting truncated chromosomes contained large amounts of endogenous chromosome sequence, including the endogenous centromere. Thus, these chromosomes were not formed *de novo*.

Hadlaczký (Mammalian Artificial Chromosomes, U.S. Patent 5,288,625 (1994)) describes a cell-line that can be used to propagate a chromosome that was formed as a result of a dicentric chromosome breakage event. All of the sequences, with the exception of a selectable marker were derived from the original, fully functional dicentric chromosome. Thus, these so called "artificial" chromosomes were not created *de novo*.

Among the "fragmentation" approaches, Haaf *et al.* (*Cell* 70:681-696 (1992)) and Praznovszky *et al.* (*Proc. Natl. Acad. Sci. USA* 88:11042-11046 (1991)) describe methods for producing chromosome fragments by integrating transfected DNA into endogenous chromosomes. Following transfection, the integrated DNA sequences become amplified (increase in copy number), and in some clones, a portion of the endogenous chromosome breaks off to produce a fragment that exists extrachromosomally. In both references, integrated transfected DNA can be found extensively on the endogenous chromosome and the extrachromosomal fragment.

In the experiments by Haaf *et al.* (*Cell* 70:681-696 (1992)), human alpha satellite DNA and the neomycin resistance gene were co-transfected into African Green Monkey cells. No other exogenous DNA was included in any of the transfections. In every transfection clone, DNA was found to be integrated into the endogenous chromosomes. In one clone, which was also found to contain an extrachromosomal fragment, the transfected alpha satellite DNA had amplified extensively following integration. The authors conclude, based on Southern blot and Fluorescence In-Situ Hybridization, that African Green Monkey sequences co-amplified with the transfected DNA and were interspersed among the alpha

satellite DNA. In further characterization of the chromosomes that contained amplified alpha satellite, it was found that "the number, size, and chromosomal location (telomeric, interstitial, or centromeric) of the transfected chromosome regions varied from cell to cell within the population of line 3-31 cells, suggesting instability of the transfected sequences." Finally, analysis of the mitotic behavior of the chromosomes containing amplified alpha satellite DNA revealed a high incidence of anaphase bridges, suggesting that the chromosomes were dicentric (or multicentric). Thus, the high degree of observed structural instability in conjunction with the high incidence of anaphase bridge structures is consistent with the idea that the chromosome fragment resulted from an integration/amplification/breakage event. Finally, it is also worth noting that in clones that contained integrated, unamplified alpha satellite DNA, no extrachromosomal fragments were observed, further suggesting that amplification is important for the chromosome fragmentation process in this method.

Praznovszky *et al.* (*Proc. Natl. Acad. Sci. USA* 88:11042-11046 (1991)) produced chromosome fragments by integrating a piece of non-centromeric human DNA (later shown to map to human chromosome 9 qter by McGill *et al.* (*Hum. Mol. Genet.* 1:749-751 (1992)) and Cooper *et al.* (*Hum. Mol. Genet.* 1:753-754 (1992)) into an endogenous chromosome. Like the Haaf experiment, the integrated transfected DNA amplified extensively, and was found to be interspersed with mouse genomic sequences. The authors suggest that the integration/amplification of the transfected DNA resulted in the formation of a dicentric chromosome that then subsequently broke to produce chromosome fragments. Analysis of the chromosome fragments shows unambiguously that the chromosome fragments were derived from the mouse chromosome containing the integrated amplified DNA.

There are a number of important similarities between the experiments by Haaf *et al.* and Praznovszky *et al.* First, both show that the transfected DNA integrated into endogenous chromosomes. Second, both show that following integration, the transfected DNA amplified extensively. Third, endogenous DNA

(untransfected chromosomal sequences from the recipient cell) was found to be interspersed throughout the amplified sequences. Fourth, the endogenous chromosomes containing the amplified transfected sequences stained with CREST antisera. Fifth, the endogenous chromosomes containing the amplified transfected sequences behaved similarly to dicentric chromosomes during mitosis. Finally, the endogenous chromosomes containing the amplified transfected sequences displayed structural instability. Thus, the large number of important similarities and the demonstrated chromosomal fragmentation by Praznovszky *et. al.* indicate a chromosome integration/amplification/breakage mechanism in both of these experiments.

Further evidence that transfection and integration of alpha satellite DNA into mammalian chromosomes is not sufficient to create extrachromosomal fragments in the absence of amplification was obtained by Larin *et. al.* (*Hum. Mol. Genet.* 3:689-95 (1994)). In these experiments, alpha satellite DNA linked to a selectable marker was transfected into human cells. In every drug-resistant clone, the alpha satellite DNA was integrated into an endogenous chromosome. While these integrations formed centromere-like structures (i.e. primary constrictions, CREST antisera staining, and lagging chromosomes during anaphase), no extrachromosomal fragments were observed in any clone. Since these experiments failed to provide clones with chromosomes containing the transfected alpha satellite DNA and not an endogenous centromere, there is no reliable method to determine whether the centromere-like structures that formed are capable of facilitating chromosome segregation.

Since each of the "pared-down" chromosomes was created from a pre-existing chromosome and since each of the "fragmentation" chromosomes was created by integrating DNA into pre-existing chromosomes, these references do not provide guidance about how to create chromosomes *de novo* from transfected naked DNA.

Furthermore, these chromosomes and the approaches used to make them have severe limitations as gene therapy vectors for several reasons. First, the

methods used to make them can only be used to create the chromosomes in cell culture. Since the breakage events are either extremely rare and/or produce chromosomes with unpredictable structure, these methods are not compatible with direct use in patients' cells. Additionally, the instability of the amplified sequences in the fragmentation approach is inconsistent with use in patients due to the risks of genomic rearrangements that, in turn, may lead to cellular transformation and cancer.

It would be highly desirable, therefore, if there were a prefabricated chromosome vector with defined structure that could be introduced directly into patient's cells, especially a vector that did not depend upon integration into endogenous chromosomes or subsequent amplification, and where the structure of the construct in the cell is substantially identical to its structure prior to transfection.

Second, pared-down chromosomes and chromosome fragments are composed of undefined endogenous sequences and provide no guidance for identifying sequences that are functionally important.

It would be highly desirable, therefore, to provide vectors composed of defined sequences and the methods to produce these defined synthetic chromosomes that allow other functionally important sequences to be rapidly identified.

Third, the chromosomes produced by the pare-down and fragmentation approaches can not be substantially purified using currently available techniques. Thus, it is difficult to deliver these pared-down chromosomes to mammalian cells without delivering other mammalian chromosomes.

It would be highly desirable, therefore, to provide substantially purified genetically engineered DNA that can be introduced into a cell and form a functional chromosome.

Fourth, since these pared-down chromosomes and chromosome fragments have never been isolated as naked DNA and reintroduced into a cell, up to the present, it was never clear whether any exogenous DNA could be introduced into

a cell to produce a functional chromosome *de novo* (without integrating into the host chromosomes first).

It would be highly desirable, therefore, to provide artificial mammalian chromosomes that are created *de novo* by introducing purified DNA into a mammalian cell.

Finally, it is very difficult to add new DNA sequences (e.g. therapeutic genes) to the pared-down chromosomes and chromosome fragments.

It would be highly desirable, therefore, to provide vectors created *in vitro*, where placing new DNA sequences onto the vectors is straight-forward and efficient.

Sun *et al.* (*Nature Genetics* 8:33-41 (1994)) describe a viral-based vector system designed for use in human cells. The vector is described as a "human artificial episomal chromosome." However, the vector relies on the presence of EBNA-1, a toxic and immunogenic viral protein. Further, the vector relies on a viral origin of replication and not on a natural mammalian chromosomal replication origin. Further, the "chromosome" does not contain functional centromeric or telomeric DNA, and does not form a functional kinetochore during mitosis. As a result, such a vector does not segregate in a controlled manner. Finally, the vector is present in the cell at an elevated copy number that ranges from 50 to 100 copies per cell, unlike endogenous chromosomes. Based on these criteria for defining mammalian chromosomes, this vector cannot be properly designated a "human artificial chromosome" because it has different properties and functions by unrelated mechanisms.

Thus, there is still a clear need for a wholly synthetic or artificial chromosome made from DNA that can be manipulated *in vitro* and, upon transfection into cells, will adopt a functional chromosome structure and will direct gene expression in a controlled manner.

The ability to clone large, highly repetitive DNA is an important step toward the development and construction of a human artificial microchromosome, and gene therapy vehicles. In addition, stable cloning of

repetitive DNA in microorganisms will be important for generating high resolution physical maps of mammalian chromosomes.

A variety of cloning systems have been developed to facilitate the cloning and propagation of foreign DNA in micro-organisms. Plasmids, bacteriophage, and yeast artificial chromosomes (YACs) have been used successfully to clone many mammalian DNA sequences. However, some types of repetitive DNA appear to be structurally unstable in these vectors (Schalkwyk *et al.*, *Curr. Opin. Biotechnol.* 6(1):37-43 (1995); Brutlag, D. *et al.*, *Cell* 10:509-519 (1977)). This results in gaps in physical genomic maps and precludes the use of these vectors as a means of propagating highly repetitive mammalian centromeric DNA.

Bacterial artificial chromosomes (BACs) have been constructed to allow the cloning of large DNA fragments in *E. coli* (O'Conner *et al.*, *Science* 244 (4910):1307-12 (1989); Shizuya *et al.*, *Proc. Natl. Acad. Sci. USA* 89(18):8794-7 (1992); Hosoda *et al.*, *Nucleic Acids Res.* 18(13):3863-9 (1990)). While this system appears to be capable of stably propagating mammalian DNA up to at least 300 kb, relatively few independent mammalian DNA fragments have been analyzed (Shizuya *et al.*, *Proc. Natl. Acad. Sci. USA* 89(18):8794-7 (1992)). In addition, the few fragments that have been tested for structural stability in the BAC vector, have not been extensively characterized with respect to the types of sequences present in each fragment. Thus, it is unknown whether these fragments contain repetitive DNA elements. In particular, it is clear, based on the restriction site and Southern analysis, that these fragments do not contain alpha satellite DNA.

Many mammalian DNA sequences appear to be structurally stable in yeast artificial chromosome (YAC) vectors, and yet certain repetitive elements of similar length are not (Neil *et al.*, *Nucleic Acids Res.* 18(6):1421-8 (1990)). Knowledge of DNA properties derived from the YAC system thus suggests that large arrays of repeating units are inherently unstable, even under conditions where similar sized DNA composed of non-repeating DNA is stable. Thus, the structural stability of large (greater than 100 kb) arrays of repeating units such as

is found in alpha satellite DNA in the BAC vector cannot be predictable with any reasonable certainty. In addition, even if some alpha satellite arrays are structurally stable in the BAC vector, *a priori*, it is not clear whether arrays of sufficient size and sequence composition to facilitate centromere function will be capable of being stably propagated in this vector.

In contrast to the cited art, several embodiments of the current invention describe a prefabricated chromosome vector with defined structure and composition that can be introduced directly into patients' cells. Since the vector described in this invention does not depend upon integration into endogenous chromosomes or subsequent amplification, the structure of the construct in the cell is substantially identical to its structure prior to transfection.

In contrast to the cited art, the vectors described in this present invention are composed of defined sequences. Furthermore, the methods used to produce these synthetic chromosomes allow other functionally important sequences to be rapidly identified.

In contrast to the cited art, with the present invention, the inventors demonstrate for the first time that artificial mammalian chromosomes can be created *de novo* by introducing purified DNA into a mammalian cell.

In contrast to the cited art, since the vectors described in the present invention are created *in vitro*, placing new DNA sequences onto the vector is straight-forward and efficient.

Summary of the Invention

It is an object of this invention to describe a method for construction of uniform or hybrid synthetic arrays of repeating DNA, and especially, alpha satellite DNA. It is a further object of the invention to describe a method for the cloning, propagation, and stable recombinant production of repeating DNA, and especially, naturally occurring or synthetic alpha satellite arrays.

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Accordingly, the invention is directed to a method for stably cloning large repeating DNA sequences, vectors containing the above arrays, and hosts transformed with such vectors.

5 The inventors have developed methods for producing large quantities of purified intact alpha satellite arrays of up to 736 kb in length. By transfecting these arrays into human cells along with telomeric DNA and human genomic DNA sequences, several wholly synthetic human chromosomes that exhibit a high degree of mitotic stability in the absence of selection have been produced.

10 Unlike previous approaches whereby attempts were made to produce an artificial mammalian chromosome, this approach does not rely on the modification of existing endogenous chromosomes. Furthermore, it does not produce multiple integration events within the endogenous chromosomes. These chromosomes were formed and maintained extrachromosomally, so integration into an endogenous chromosome is avoided.

15 The relatively high frequency of synthetic chromosome formation and the lack of other genomic rearrangements associated with the chromosome formation, allows the synthetic chromosomes made by the inventors to be used as effective vectors for heterologous gene expression and gene therapy.

20 The invention is thus based on the inventors' discovery that by means of isolated purified DNA alone, a synthetic or artificial chromosome is produced *de novo* (from purified DNA) in a cell and is produced and maintained as an extrachromosomal element. This chromosome retains the essential functions of a natural mammalian chromosome in that it is stably maintained as a non-integrated construct in dividing mammalian cells without selective pressure, just
25 as naturally-occurring chromosomes are inherited. For a linear chromosome, this indicates centromeric, telomeric, and origin of replication functions.

30 The invention is thus directed to a synthetic or artificial mammalian chromosome. The chromosome is produced from isolated purified DNA. The isolated purified DNA is transfected into mammalian cells. Without integrating into an endogenous chromosome, it forms a functional chromosome. This

chromosome is not derived from an endogenous naturally-occurring chromosome *in situ*. The starting material is isolated purified centromeric DNA and DNA that allows chromosome formation without integration. For linear chromosomes, telomeric DNA is included. In a preferred embodiment, the DNA that allows chromosome formation without integration is genomic DNA (from the naturally-occurring genome of an organism).

The artificial mammalian linear chromosome thus preferably essentially comprises centromeric, telomeric, and genomic DNA. In one embodiment, the artificial chromosome is a circular chromosome. In this case, telomeric DNA is absent since it is not necessary to replicate chromosome ends.

The genomic DNA is a subgenomic DNA fragment that is a restriction enzyme digestion fragment, a fragment produced by mechanical shearing of genomic DNA, or a synthetic fragment synthesized *in vitro*. The genomic DNA starting material (ie., that is transfected) can be a mixture of heterogeneous fragments (e.g., a restriction digest) or can be a cloned fragment or fragments (homogeneous).

Centromeric DNA comprises a DNA that directs or supports kinetechore formation and thereby enables proper chromosome segregation. Centromeric DNA at active, functional, centromeres is associated with CENP-E during mitosis, as demonstrated by immunofluorescence or immunoelectron microscopy. By "associated" is meant that the centromeric DNA and CENP-E co-localize by fluorescence *in situ* hybridization (FISH) and immunofluorescence.

Telomeric DNA comprises tandem repeats of TTAGGG that provide telomere function, i.e., replicate the ends of linear DNA molecules. Telomeric DNA is included as an optional component, to be used when linear chromosomes are desired. This is indicated herein by enclosing the terms "telomeric"/"telomere" in parentheses.

Prior to transfection, the DNA can be naked, condensed with one or more DNA-condensing agents, or coated with one or more DNA-binding proteins.

The invention is also directed to an artificial mammalian chromosome produced by the process of introducing into a mammalian cell the isolated purified DNA fragments above. In a preferred embodiment the process uses DNA essentially comprising centromeric, telomeric, and genomic DNA.

5 The various fragments can be transfected separately or one or more can be ligated prior to transfection. Thus the centromeric (telomeric) and genomic DNAs are introduced separately (unligated) or one or more of the isolated purified DNAs are ligated to one another.

10 The invention is also directed to a mammalian cell containing and compositions comprising the artificial mammalian chromosome.

The invention is also directed to the isolated purified DNA described above, and which forms an artificial mammalian chromosome when introduced into a mammalian cell. In preferred embodiments, the isolated purified DNA essentially comprises centromeric, telomeric, and genomic DNA.

15 The invention is also directed to a mammalian cell containing and compositions comprising the purified DNA.

The invention is also directed to a vector or vectors containing the purified DNA.

20 The invention is also directed to a mammalian cell containing and compositions comprising the vector(s).

25 The invention is also directed to the isolated purified DNA described above produced by the process of combining one or more of the DNAs described above. In preferred embodiments, the DNA includes: (1) centromeric DNA, (2) telomeric DNA, (3) genomic DNA. The DNAs can be unligated or one or more can be ligated to one another.

The invention is also directed to a method for making an artificial mammalian chromosome by introducing into a mammalian cell the purified DNA described above.

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The invention is also directed to a method for making DNA capable of forming an artificial chromosome, the method comprising combining *in vitro* the DNA described above.

5 The invention is also directed to a method for propagating an artificial chromosome in mammalian cells by introducing the purified DNA into a mammalian cell and allowing the chromosome to replicate.

In a preferred embodiment, the invention is also directed to methods for expressing a heterologous gene in a mammalian cell by expressing that gene from the artificial mammalian chromosome.

10 Thus, the invention is also directed to methods for providing a desired gene product by including a desired gene on the artificial chromosome such that the gene of interest is expressed. In preferred embodiments, the invention provides a method of gene therapy by including heterologous therapeutic DNA on the artificial mammalian chromosome, such that there is a therapeutic effect
15 on the mammal containing the chromosome.

In a preferred embodiment of the invention, the centromeric DNA is alpha-satellite DNA.

20 In a preferred embodiment of the invention, the artificial mammalian chromosome is derived entirely from human DNA sequences and is functional in human cells.

Brief Description of the Figures

25 **Figure 1** is a schematic diagram of the method of the invention. The numbers "1-16" represent 1-16 copies of monomeric units (of approximately 171 bp) of alpha satellite DNA, tandemly aligned in a linear array. "X" represents a desired restriction enzyme site in the backbone of the vector carrying the array during its expansion to a desired size.

Figure 2 is a graphical representation of the correlation of the percent recombinants (after 50 generations) and the array size (kb).

Figure 3. Method for producing large head-to-tail tandem arrays of alpha satellite DNA. pVJ104-Y α 16 was linearized with *Bam*HI and *Sfi*I, and purified by pulsed field gel electrophoresis (PFGE). Likewise, pBac-Y α 16 was linearized with *Bam*HI and *Bgl*II and the alpha satellite array was purified by PFGE. A) The purified arrays were incubated together in the presence of ligase, *Bam*HI and *Bgl*II. Since *Bam*HI and *Bgl*II are complementary/nonisoschisomeric overhangs, a ligation event resulting in a *Bam*HI/*Bgl*II junction (as is the case in a head-to-tail joining) will destroy both sites. Thus, a head-to-tail junction will be resistant to cleavage by *Bam*HI and *Bgl*II. In contrast, a head-to-head, or tail-to-tail ligation event will recreate a *Bam*HI or *Bgl*II site, respectively. Since *Bam*HI and *Bgl*II are present, these ligation products will be cleaved to produce their constituent monomers (or head-to-tail multimers). By controlling the amount of ligase, the incubation time, and the concentration of DNA, the length of the head-to-tail products can be varied as necessary. B) Following ligation, the products were analyzed by PFGE. Lane 1, molecular weight standards (NEBL Midrange II markers); lane 2, Y α 16 (*Bam*HI/*Bgl*II fragment) ligated in the presence of *Bam*HI and *Bgl*II for 4 hours; lane 3, Y α 16 (*Bam*HI/*Bgl*II fragment) ligated in the presence of *Bam*HI/*Bgl*II for 12 hours; lane 4, Y α 16 (*Bam*HI/*Bgl*II fragment) mock-ligated in the presence of *Bam*HI and *Bgl*II; lane 5, VK75 (*Bss*HII fragment) ligated for 12 hours without restriction enzyme; lane 6, VK75 (*Bss*HII fragment) ligated for 12 hours in the presence of *Bss*HII; lane 7, VK75 (*Bss*HII fragment) mock-ligated. The molecular weight of ligation products are shown on the left. Note: Although these samples were run on the same gel, several irrelevant lanes between lanes 4 and 5 were removed.

Figure 4. Strategy for making synthetic chromosomes.

Figure 5. Analysis of synthetic chromosomes from clones 22-7 and 22-13 by fluorescent *in situ* hybridization (FISH). Cells were harvested, dropped onto glass slides, and hybridized to Y alpha satellite DNA as described in the

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Experimental Procedures (See Examples herein). The biotinylated probe was detected using Texas Red Avidin and amplified with two layers of biotinylated anti-Avidin and Texas Red Avidin. A) DAPI image of a metaphase spread from clone 22-7. B) Same as A) except that the alpha satellite probe was visualized using a triple cube filter. C) DAPI image of a metaphase spread from clone 22-13. D) Same as C) except that the alpha satellite probe was visualized using a triple cube filter. In each case, the synthetic chromosome is indicated with a white arrow.

Figure 6. Analysis of synthetic chromosomes from clones 22-6 and 23-1 by FISH. Cells were harvested, dropped onto glass slides, and hybridized to Y alpha satellite DNA (clone 22-6) or 17 alpha satellite DNA (clone 23-1) as described in the experimental procedures. The biotinylated probe was detected using Texas Red Avidin and amplified with two layers of biotinylated anti-Avidin and Texas Red Avidin. A) DAPI image of a metaphase spread from clone 22-6. B) Same as A) except that the alpha satellite probe was visualized using a triple cube filter. C) DAPI image of a metaphase spread from clone 23-1. D) Same as C) except that the alpha satellite probe was visualized using a triple cube filter. In each case, the synthetic chromosome is indicated with a white arrow. In D), the yellow arrow indicates the location of the C group chromosome at the integration site.

Figure 7. Analysis of synthetic chromosomes from clones 22-11 and 17-15 by FISH. Cells were harvested, dropped onto glass slides, and hybridized to Y alpha satellite DNA (clone 22-11) or 17 alpha satellite DNA (clone 17-15) as described in the experimental procedures. The biotinylated probe was detected using Texas Red Avidin and amplified with two layers of biotinylated anti-Avidin and Texas Red Avidin. A) DAPI image of a metaphase spread from clone 22-11. B) Same as A) except that the alpha satellite probe was visualized using a triple cube filter. C) DAPI image of a metaphase spread from clone 17-15. D) Same

as C) except that the alpha satellite probe was visualized using a triple cube filter. In each case, the synthetic chromosome is indicated with a white arrow.

Figure 8. Determination of the amount of transfected alpha satellite DNA present in clones containing the synthetic chromosome. A) Total genomic DNA was harvested, digested, and electrophoresed as described in the Experimental Procedures. Lane 1, HT1080; lane 2, clone 22-6; lane 3, clone 22-7; lane 4, clone 22-11; lane 5, clone 22-13; lane 6, clone 23-1. B) The estimated amount of synthetic Y alpha satellite DNA is shown for each clone. Note: clone 23-1 was transfected with 17 alpha satellite DNA, and therefore, does not contain synthetic Y alpha satellite DNA.

Figure 9. CENP-E is associated with the synthetic chromosomes during mitosis. Immunofluorescence was carried out on metaphase chromosomes harvested from synthetic chromosome-containing clones as described in experimental procedures. A) DAPI- stained chromosomes from clone 22-11. B) Same as A) except the location of the anti-CENP-E antibodies is visualized using a triple cube filter. C) DAPI- stained chromosomes from clone 23-1. D) Same as C) except the location of the anti-CENP-E antibodies is visualized using a triple cube filter. In each case, the synthetic chromosome is indicated by a white arrow.

Figure 10. X-Gal plate staining of clone 22-11 after growth for 70 days in the absence of selection. Cells were harvested and stained as described in the Experimental Procedures herein. A) HT1080 B) Clone 22-11. The presence of blue cells in clone 22-11, but not in HT1080 indicates that β -geo is still expressed in these cells.

Detailed Description of the Preferred Embodiments

The inventors have discovered that functional mammalian chromosomes can be constructed from purified DNA introduced into a mammalian cell. There are several advantages to using these chromosomes for a variety of applications.

5 First, since they are formed and replicate autonomously, they will not result in insertional mutagenesis by inserting into the host genome.

Second, because of the large size of the transfer vector (in the megabase range), there is the capacity to accommodate the entire repertoire of a large gene including all of its regulatory elements. This itself may encompass megabases
10 of DNA.

Third, because some genetic diseases are the result of defects in more than one gene, because of the large size of the mammalian artificial chromosome more than one gene can be accommodated.

Fourth, the chromosomes are stable and can thus provide a therapeutic
15 benefit over many cell divisions.

Fifth, the chromosomes are non-immunogenic.

The method of the invention thus provides a method in microorganisms for producing structurally intact highly repetitive regions of DNA, which are utilized in the construction of artificial chromosomes. Arrays of defined length, composition, orientation and phasing are possible. By "proper phasing" is meant
20 that the precise length and orientation of any given higher order repeat in the array is not altered from that in the naturally-occurring sequence by the construction of the array, and also that there are no non-repeating DNA at the junction of the repeating units. For alphoid sequences, for example, the length and orientation of the higher order repeating unit is exactly the same as the
25 naturally-occurring higher order repeat, and there are no non-alphoid sequences present at the junction of the higher order repeats, except for the bases modified to create the restriction sites.

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Accordingly, a method for cloning repeating tandem arrays of DNA is provided, wherein a first DNA unit is prepared such that the opposing ends of the DNA unit contain complementary, but non-isoschizomeric restriction sites. This DNA is ligated into a vector, and the vector is linearized at one of the restriction sites. A second DNA unit, prepared as in step (a), is then ligated in tandem with the first unit, so as to form a directional, repeating array. This array is transformed into a host cell, and especially a bacterial host cell, and stable clones containing the array are selected. Starting with the vector linearization, these steps are repeated until a desired array size is reached.

The directional cloning scheme of the invention is illustrated in Figure 1, in which the cloning of alpha satellite DNA higher order repeats is illustrated. As shown in the figure, the method of the invention utilizes a "build-up" approach, in which shorter units, preferably higher order repeats, are added together to create the longer tandem array of repeating units. The units are added to each other in a manner that results in a defined orientation, which is established by two different restriction sites - one at each end of each repeating unit. Preferably, the repeating unit, and especially the higher order repeating unit, that is the basis of the tandem array, contains complementary, but non-isoschizomeric restriction sites at opposing ends. Such ends may be designed into the unit using methods such as polymerase chain reaction. In the method of the invention, by complementary ends, it is intended to include both complementary overhanging ends and blunt ends.

In a preferred embodiment, the DNA array is alphoid DNA. As shown in Example 1, polymerase chain reaction can be used to amplify a single 2.7 kb DNA alphoid unit (actual length 2.712 kb) such that complementary restriction sites (*Bam*H I and *Bgl* II) are created at opposing ends of the higher order register of alpha satellite repeat from human chromosome 17. The ends of the higher order repeats can be modified using polymerase chain reaction-mediated site directed mutagenesis so that complementary restriction sites are created at opposite ends of each repeat. The modified higher order repeats are then cloned

into the mini-F cloning vector pBAC108L (Shizuya, H. *et al.*, *Proc. Natl. Acad. Sci. USA* 89:8794-8797 (1992), incorporated herein by reference). These complementary restriction sites are used in conjunction with non-complementary flanking restriction sites to directly clone synthetic arrays of alpha satellite DNA derived from a single modified higher order repeat (Figure 3). Synthetic arrays can be created from any higher order alphoid repeat, including such alphoid DNA derived from chromosome 17, the Y chromosome, or other chromosome. In addition, hybrid arrays consisting of higher order repeats from both chromosomes can be prepared. In a preferred embodiment, the DNA is human DNA.

Arrays up to 200-215 kb are stable in the vector and hosts of the invention. In one embodiment, an array of 87 kb - 215 kb in length is constructed. In a preferred embodiment, an array of at least 100 kb in length is constructed. In a highly preferred embodiment, an array of at least 140 kb, and especially at least 174 kb in length is constructed; arrays of 174 kb exceed the minimum known observed length of a functional alpha satellite array.

Examples of useful complementary, but non-isoschizomeric restriction enzymes that are useful in creating such sites include: *Sal* I and *Xho* I; *Mun* I and *EcoR* I; *Afl* III and *Nco* I/*Sty* I (isoschizomers: either one can be the partner for the non-isoschizomer partner); *Nhe* I and *Xba* I and *Sty* I/*Avr* II (isoschizomers) and *Spe* I (any combination); *Cla* I and *BstB* I and *Acc* I (any combination); *Mlu* I/*Afl* III (isoschizomers) and *BssH* II and *Asc* I; and *Not* I and *Eag* I. *Bcl* I is a complementary/non-isoschizomer of both *BamH* I and *Bgl* II.

The amplified DNA is then digested using, for example, (1) *BamH* I and *Sfi* I, or (2) *Bgl* II and *Sfi* I. Following separation of the bands in the digested DNA using physical methods capable of separating such DNA, such as, for example, gel electrophoresis, the DNA band from one digest is excised and ligated to the excised DNA band from the other digest. In the above example, since *Bgl* II and *BamH* I generate compatible overhangs, and *Sfi* I generates an asymmetric overhang that can only relegate in a particular orientation, DNA

flanked by these sites ligates to the vector DNA to create a tandem dimer arrayed in head to tail fashion. This DNA can then be transformed into a microorganism.

In a second variation of this strategy, blunt cutting restriction enzymes can be substituted for *Bam*H I and *Bgl* II. For example, *Sma* I and *Eco*R V can be substituted for *Bam*H I and *Bgl* II, respectively. The digestion and fragment isolation are then carried out as above. An important feature of this strategy, common to both the blunt and complementary/nonisoschizomeric variations, is that the physiologic phasing of the arrays can be precisely maintained, if desired. Examples of additional blunt cutters that can be used include *Ssp* I, *Stu* I, *Sca* I, *Pml* I, *Pvu* II, *Ecl* 136 II, *Nae* I, *Ehe* I, *Hinc* II, *Hpa* I, *Sna*B I, *Nru* I, *Fsp* I, *Dra* I, *Msc* I, *Bst* 1071, *Alu* I, *Asp* 700/*Xmn* I, *Avi* II, *Bbr*P I, *Bst* 1107, *Eco*47 III, *Dpn* I, *Hae* III, *Hind* II, *Nam* I, *Mlu*M I, *Mvn* I, *Rsa* I, *Swa* I, *Bsh* 1236 I, *Eco*72 I, *Pal* I, and *Srf*I.

Structural stability of the cloned plasmids containing large tandem arrays of synthetic alpha satellite, in microorganisms, DNA can be determined using simple growth and dilution experiments as described in Example 2. For example, structural stability can be determined by passage for 50 generations and subsequent analysis of plasmid DNA for structural integrity. Plasmid structure can be analyzed by restriction analysis, and agarose gel electrophoresis. Little or no recombination was observed for these clones, indicating that the directional cloning scheme can be employed to construct and propagate synthetic alpha satellite arrays in the context of a mini-F cloning vector (such as the pBAC108L vector) and a suitable *E. coli* host.

The method of the invention can be used to construct any desired repeating DNA unit. For example, alpha satellite DNA from any eukaryotic chromosome, especially human DNA, can be cloned. Other examples of large tandem arrays of highly repetitive DNA include the immunoglobulin DNA loci, regions of heterochromatic repeats, and telomeres.

The directional cloning method of the invention does not require the presence of polymorphic restriction sites, as required when cloning endogenous

(nonmodified) arrays. Even when present, these sites do not permit control of the exact size of the array. Furthermore, by using a single higher order repeat as used in the method of the invention, (see Figure 1), and sequentially doubling its size, the exact sequence of the entire array is known, since the sequence of the original higher order repeat is known.

When cloning endogenous arrays, such as, for example, endogenous alpha satellite arrays, one cannot be confident of the precise composition of a given array. In particular, interruptions in the arrays by non-repetitive DNA may have significant effects on stability in *E. coli*. In addition, to be suitable as a vector for gene therapy, one must know the exact sequence of the vector being provided to the recipient of such therapy. The method of the invention obviates that concern and allows the artisan to bypass sequencing of native alpha satellite arrays, in favor of constructing a useful array, *de novo*, from known repeating sequences.

Structurally diverged higher order repeats generally exhibit increased structural stability in *E. coli* relative to more homogeneous arrays. Thus, by utilizing homogeneous synthetic arrays according to the method of the invention, an accurate determination of the minimal stability of the repeating DNA in the vector can be obtained.

Any desired bacterial host in which the vector is stably maintained may be used as the host. Especially *E. coli* is useful when utilizing BAC vectors and the BAC system.

Synthetic alpha satellite arrays can be utilized in the construction of synthetic human chromosomes in the following manner: (1) by transfection of synthetic alpha satellite arrays into a human or other mammalian cell line; (2) by transfection of synthetic alpha satellite arrays in conjunction with randomly cloned human DNA or specific DNA fragments, into a human or other mammalian cell line; (3) by co-transfection into a human or other mammalian cell line of synthetic alpha satellite arrays with unlinked specific chromosomal components, such as telomeric DNA, matrix attachment regions, and/or other chromosomal loci that enhance the mitotic stability of alpha satellite-containing

episomal DNA. Co-transfection of these components in unlinked form allows the transfected cell line to construct an infinite number of structural permutations, permitting the most mitotically stable forms to be retained, while the unstable forms are lost over time. Stable conformations can subsequently be harvested
5 utilizing standard methods and procedures. Those constructs that exhibit mitotic stability in the absence of selective pressure can be isolated and subsequently utilized in the preparation of gene therapy vectors containing one or more therapeutically useful entities such as genes, ribozymes, or antisense transcripts.

The invention is thus directed to a synthetic or artificial mammalian
10 chromosome comprising essentially centromeric, genomic, and optionally, telomeric DNA. In an alternative embodiment, the artificial chromosome is a circular chromosome. In this case, telomeric DNA is absent since it is not necessary to replicate chromosome ends. The chromosome has, at the minimum, DNA sequences that provide essential chromosomal functions in a mammalian
15 cell.

The genomic DNA is a subgenomic DNA fragment selected from the group consisting of restriction enzyme digestion fragments mechanically sheared fragments, and fragments of DNA synthesized *in vitro*. The genomic DNA component of the chromosome can be derived from a mixture of subgenomic
20 fragments (e.g., a restriction enzyme digest) or from cloned fragment(s).

The function of the genomic DNA is two-fold. The DNA expresses a gene product, or causes the expression of a gene product (as, for example, by having a regulatory function), and the DNA allows the formation of an artificial chromosome from purified DNA in a cell without the integration of the purified
25 DNA into an endogenous chromosome in the cell, the artificial chromosome also containing centromeric DNA and, optionally, telomeric DNA. The genomic DNA can be derived from any organism and can be of any size.

The genomic DNA that forms a component of the synthetic mammalian chromosome may be derived from a mammalian source other than the mammal
30 from which the cell is derived in which the chromosome replicates. For example,

mouse genomic DNA can be provided to human cells and human genomic DNA can be provided to the cells of other mammals. Further, it can be from a source different from the source of the centromere or telomere.

5 Still further, the function of the genomic DNA exemplified herein can be potentially carried out by genomic DNA of any organism, including procaryotic organisms, and by DNA synthesized *in vitro* and not corresponding to a naturally-occurring sequence, partly homologous to a naturally occurring sequence, or completely non-homologous.

10 Centromeric DNA essentially comprises a DNA that directs or supports kinetechore formation and thereby enables proper chromosome segregation. This centromeric DNA at active, functional centromeres is associated with CENP-E during mitosis, as demonstrated by immunofluorescence or immunoelectron microscopy. By "associated" is meant that the centromeric DNA and CENP-E co-localize by FISH and immunofluorescence.

15 In a preferred embodiment of the invention, the centromeric DNA is alpha satellite DNA. However, any functional centromeric DNA, and especially repetitive DNA, is enabled by the methods described herein and useful for making artificial chromosomes.

20 The inventors have created *in vitro* methods for producing large alpha satellite arrays. Previously, no method has been available allowing structurally intact alpha satellite DNA greater than 200 kb to be purified in the quantities necessary for the transfection of mammalian cells. By using these methods, controlled amounts of alpha satellite DNA can be produced *in vitro*. As described herein, by empirically controlling the amount of ligase, incubation time, and
25 concentration of DNA, the length of the ultimate product can be varied as necessary.

30 However, the invention is not limited to centromeric DNA derived from alpha satellite DNA. The *in vitro* methods created by the inventors can be applied to any centromeric DNA that functions as described herein, and especially to repetitive DNA.

Further, the entire alpha satellite repeat may not be required for centromere formation. Thus, the centromeric DNA can also comprise alpha satellite derivatives and analogs, for example, sub-monomer regions in alpha satellite or related satellite DNA.

5 Subregions within the alphoid monomer representing protein binding sites can be ligated together to generate a functional centromere, consisting of a smaller repeat unit. The functionality of this embodiment is shown by data from mouse-human hybrids.

10 In the murine species *M. musculus*, minor satellite DNA contains CENP-B boxes and appears to be the functional equivalent of alpha satellite DNA. Interestingly, in *M. musculus*, the minor satellite repeat unit is only 120 bp and has no apparent sequence homology to alpha satellite DNA outside of the CENP-B box. Despite the difference in repeat size and sequence, human chromosomes segregate efficiently in mouse/human hybrids. This demonstrates
15 that the centromeric repeat unit size and sequence can vary without destroying centromere function.

The murine species *M. caroli* apparently lacks minor satellite DNA (Kipling *et al.*, *Mol. Cell. Biol.* 15:4009-4020 (1995)). In this species, the functional alpha satellite equivalent appears to be a 79 bp satellite sequence that
20 contains a CENP-B box (there is also a 60 bp sequence that is 97% homologous to the 79 bp sequence but that lacks a CENP-B box). In crosses between *M. musculus* and *M. caroli*, chromosomes from both species segregate normally within the same cell. This shows that both the minor satellite and the 79 bp satellite sequences are recognized by the same spindle during mitosis. Thus,
25 different centromeric repeat sizes can be functional.

Since alpha satellite, minor satellite, and 79 bp satellite repeats are different sizes and are functional, the absolute repeat size *per se* is not the determinant of functionality of centromeric DNA. Additionally, since there is only limited sequence homology between these centromeric repeats, it is likely

that subregions within the repeats representing protein binding sites are the important functional component.

Thus, in one embodiment of this invention, the centromeric DNA contains subregions within alpha satellite DNA. In a preferred embodiment, the centromeric DNA is composed of tandemly ligated CENP-B boxes, defined by the sequence 5'a**TTCG**ttggAaa**CGG**Ga3' (SEQ ID. NO.:1), where the bases indicated by capital/bold letters are the most important for CENP-B binding and the bases indicated by lower case letters may be substituted with other bases.

In other embodiments, alphoid equivalents from other species are used for centromeric DNA. Human and other mammalian chromosomes have been shown to segregate efficiently in cells from other species as demonstrated by interspecies somatic cell hybrids. Examples of these hybrids include mouse x human, hamster x human, rat x human, hamster x mouse, rat x mouse, and chicken x human. The ability of a human chromosome to segregate in chicken cells (Dieken, E., *et al.*, *Nature Genet.* 12:174-182 (1996)) shows that human centromeric DNA is also functional in a non-mammalian species (i.e., avian).

Based on observations from cross-species hybrids, it is clear that chromosomes from one species are functional in other species. Therefore, synthetic chromosomes can be produced in human cells using centromeric repeats from other mammals (and avians) instead of, or in conjunction with, alpha satellite DNA. Conversely, alpha satellite DNA can be used as the source for centromeric DNA in other mammalian (and avian) species.

Thus, in a further embodiment of the invention, genomic (telomeric) DNA is transfected into cells along with *M. musculus* minor satellite DNA, *Mus caroli* 79 bp satellite DNA, or analogous sequences from other mammals. In another embodiment, telomeric and genomic DNA is transfected into cells along with centromeric DNA from avian cells.

Essentially, centromeric DNA that is associated with CENP-E during mitosis is embodied in the aspect of the invention that encompasses the use of centromeric sequences heterologous to the host cell and other synthetic

chromosomal components. As long as the centromeric sequence in the chromosome is associated with CENP-E during mitosis, a functional chromosome for mammalian cells would be expected to result irrespective of the genomic sequence(s) and telomere sequences, and for that matter, irrespective of the specific centromeric sequence.

The telomeric DNA can be derived from any DNA sequence (from any desired species) that retains a telomeric function. In mammals and other vertebrates, the most abundant and conserved sequence at the chromosome end is TTAGGG, which forms arrays between 2 and 20 kilobases in length. Human telomere DNA consists of about 5 kilobases of the repeat TTAGGG, and small stretches of this sequence are enough to seed telomere formation after introduction of linear molecules into mammalian cell lines (Huxley, C., *Gene Ther.* 1:7-12 (1994)). Simple (TTAGGG)_n arrays are sufficient to provide the telomere function required by an artificial chromosome. The telomeric DNA, therefore, comprises tandem arrays of the hexamer TTAGGG. Telomeric DNA is included when the formation of linear chromosomes is desired.

Telomeres, centromeres and replication origins are discussed in Huxley, C. *et al.*, *Biotechnol.* 12:586-590 (1994).

The invention is also directed to purified DNA molecules that essentially comprise centromeric, genomic, and optionally, telomeric DNA, as described herein.

In one embodiment, the purified DNA is naked DNA.

In another embodiment, the purified naked DNA is condensed with one or more agents that condense DNA. It may be advantageous to condense the purified DNA prior to transfection in order to stabilize it against shearing. By condensing the purified centromeric (telomeric) and genomic DNA prior to transfection, it will become more resistant to structural insult arising from manipulations during transfection. Thus, in one embodiment of this invention, the purified centromeric (telomeric) and genomic DNA is condensed with one or more DNA condensing agents prior to transfection. In this respect, polycations

have been shown to physically condense high molecular weight DNA and to protect it from mechanical shearing (Kovacic *et al.*, *Nucleic Acids Res.* 23:3999-4000 (1995); Widom and Baldwin, *J. Mol. Biol.* 144:431-453 (1980); Widom and Baldwin, *Biopolymers* 22:1595-1620 (1983)). Therefore, in a further
5 embodiment, the purified DNA is condensed with polycationic compounds. Examples of polycationic compounds include poly-lysine, poly-arginine, spermidine, spermine, and hexaminecobalt chloride.

In an alternative embodiment, the invention encompasses precoating DNA with proteins. It may also be advantageous to precoat the DNA with DNA-
10 binding proteins such as histones, nonhistone chromosomal proteins, telomere binding proteins, and/or centromere binding proteins. This precoating is expected to have several desirable consequences. First, it will result in condensation of the DNA which will protect the high molecular weight DNA from shearing. Second, it will inhibit nuclease degradation of the transfected DNA by blocking nucleases
15 from binding to the DNA. Third, the precoated DNA may enter the nucleus more efficiently following transfection, since each of the proteins listed above contain nuclear localization signals. By precoating the centromeric (telomeric) and genomic DNA with DNA binding proteins prior to transfection, we expect to increase the efficiency of transfection and synthetic chromosome formation.

20 Thus, in another embodiment of this invention, the purified centromeric (telomeric) and genomic DNA is coated with DNA binding proteins prior to transfection. Examples of DNA-binding proteins include histones, non-histone chromosomal proteins, transcription factors, centromere binding proteins, and telomere binding proteins.

25 DNA-binding proteins can also be identified and purified by their affinity for DNA. For example, DNA binding may be revealed in filter hybridization experiments in which the protein (usually labeled to facilitate detection) is allowed to bind to DNA mobilized on a filter or, vice-versa in which the DNA binding site (usually labeled) is bound to a filter upon which the protein has been
30 immobilized. The sequence specificity and affinity of such binding is revealed

with DNA protection assays and gel retardation assays. Purification of such proteins may be performed utilizing sequence-specific DNA affinity chromatography techniques, for example column chromatography with a resin derivatized with the DNA to which the domain binds. Proteolytic degradation of DNA-binding proteins may be used to reveal the domain which retains the DNA binding ability.

The invention is thus directed to an artificial mammalian chromosome produced by the process of transfecting a mammalian cell with the purified DNA, described herein, and allowing the cell to completely reconstitute the DNA *in vivo*.

The invention is thus directed to an artificial mammalian chromosome produced by the process of transfecting a mammalian cell with purified naked DNA, the DNA comprising essentially centromeric DNA (telomeric DNA) and genomic DNA, as described herein.

The invention is thus also directed to an artificial chromosome produced by the process of transfecting a mammalian cell with purified condensed DNA, the DNA comprising essentially, centromeric DNA (telomeric DNA), and genomic DNA, as described herein.

The invention is thus also directed to an artificial mammalian chromosome produced by the process of introducing purified coated DNA into a mammalian cell, the DNA comprising essentially a centromere (a telomere) and genomic DNA, as described herein.

The invention is also directed to purified DNA made by the process of combining, *in vitro*, isolated purified and genomic DNA (telomeric DNA) as described herein.

The invention is also directed to purified, condensed DNA made by the process of combining, *in vitro*, isolated purified centromeric DNA (telomeric DNA) and genomic DNA, as described herein. Alternatively, the individual DNA components could be pre-condensed and then combined.

The invention is also directed to purified, coated DNA made by the process of combining, *in vitro*, isolated purified centromeric DNA (telomeric DNA) and genomic DNA, as described herein and adding DNA-binding proteins. Alternatively, the individual DNA components could be pre-coated and then combined.

The purified DNA described above may comprise unligated centromeric (telomeric) and genomic DNA. Alternatively, the purified DNA described above can also comprise centromeric (telomeric) and genomic DNA in which one or more of these DNAs are ligated to each other.

The invention is also directed to a composition comprising the purified DNA described above. The composition may contain components that facilitate the entry of the DNA into a cell. For the formation of an artificial chromosome, the composition may facilitate the uptake of the DNA into a mammalian cell. Alternatively, the composition may comprise ingredients that facilitate the uptake of the DNA into a cell which is used for propagation of a vector containing the DNA.

The invention is also directed to a vector containing the DNA described above. The vector may be used for propagating the DNA, i.e., amplifying the sequences described above prior to introducing them into a mammalian cell and forming an artificial chromosome.

Accordingly, the invention is also directed to a composition comprising the vector containing the DNA described above.

The invention is also directed to a cell containing the vector described above.

The invention is also directed to a mammalian cell containing the artificial chromosome.

The invention is also directed to a mammalian cell containing the purified DNA described above.

Although any mammalian cell is encompassed by the invention, in preferred embodiments of the invention, the mammalian cell is a human cell.

In preferred embodiments of the invention, the centromeric DNA is human alpha satellite DNA. It is understood, however, that alpha satellite DNA may be derived from any primate. The invention further encompasses centromeric DNA from non-primate mammals, wherein said centromeric DNA is associated with CENP-E during mitosis. Any centromeric DNA that is associated with CENP-E during mitosis, and especially repetitive DNA, irrespective of the organism from which it is derived, is expected to provide functional centromeric sequences for an artificial mammalian chromosome according to the present invention. Thus, an artificial mammalian chromosome that functions in human cells, for example, may contain centromeric sequences derived not from humans but from non-human mammals and even from non-mammalian species such as avians. Any repetitive DNA that is associated with CENP-E is potentially useful. Accordingly, following the methods taught herein, any centromeric sequence can be tested for function as a component of a mammalian artificial chromosome.

In specific disclosed embodiments of the invention, the centromeric DNA comprises large stretches of alpha satellite array, a segment composed of the repeating telomeric sequence (TTAGGG)_n, and random genomic fragments produced by digestion with the restriction enzyme *NotI*. In preferred embodiments, the restriction enzyme digests DNA into pieces in the range of fragments generated by *NotI* digestion of human genomic DNA and preferably in the range of 10 kb to 3 mb. This includes but is not limited to *BamHI*, *BglI*, *Sall*, *XhoI*, *SfiI*, *NotI*, *SrfI*, *PmeI*, and *Ascl*.

When the purified DNA is introduced into a mammalian cell, this DNA forms a functional synthetic or artificial chromosome. This chromosome has the characteristics of a naturally-occurring mammalian chromosome. The chromosome is present in the cell at a low copy number, usually one per cell. The chromosome is linear and contains telomeric sequences. CENP-E is associated with the artificial chromosome during mitosis, indicating the formation of a functional kinetochore. The chromosome is mitotically stable in the absence

of selection. The chromosome is structurally stable with time with an undetectable integration frequency. The chromosome contains one or more transcriptionally active genes. Thus, these chromosomes do not originate from naturally-occurring chromosomes but are constructed starting *in vitro* from isolated purified DNA sequences.

Accordingly, the invention is also directed to a method for making an artificial mammalian chromosome, the method comprising introducing into a mammalian cell the purified DNA described above.

The DNA can be introduced into the mammalian cell by any number of methods known to those skilled in the art. These include, but are not limited to, electroporation, calcium phosphate precipitation, lipofection, DEAE dextran, liposomes, receptor-mediated endocytosis, and particle delivery. The chromosomes or DNA can also be used to microinject eggs, embryos or *ex vivo* or *in vitro* cells. Cells can be transfected with the chromosomes or with the DNA described herein using an appropriate introduction technique known to those in the art, for example, liposomes. In a preferred embodiment of the invention, introduction of purified DNA into the mammalian cell is by means of lipofection.

The purified DNA is thus useful for transfecting a mammalian cell, said transfecting resulting in the formation of an artificial chromosome in the cell from the transfected DNA.

The DNA can be propagated in non-mammalian cells separately or where one or more of the components is ligated together. Thus, the invention is also directed to the purified DNA ligated into a vector for propagation. Such vectors are well-known in the art and include, but are not limited to, pBac108L, P1, pACYC184, pUC19, pBR322, YACs, and cosmids.

The invention is also directed to a mammalian cell containing the artificial or synthetic chromosome. The invention is directed to any mammalian chromosome or mammalian cell. Although all mammals are encompassed, the preferred embodiment is the human. A preferred embodiment of the invention therefore encompasses a human cell containing a synthetic human chromosome.

The DNA and chromosomes have been developed especially for use as expression vectors for gene therapy and other purposes. Therefore, in preferred embodiments of the invention, the purified DNA also consists essentially of one or more DNA sequences useful for the expression of a desired gene product, for example, as therapeutic agents. The invention is thus directed to a method for introducing expressible DNA into a cell by including this DNA on the artificial chromosome. The DNA can be regulatory, structural, expressed as a gene product, and the like. In a preferred embodiment, the DNA provides a gene product. When transfected into mammalian cells, the artificial chromosomes that are formed following transfection harbor and express these DNA sequences.

Recombinant DNA technology has been used increasingly over the past 20 years for the production of desired biological materials. DNA sequences encoding a variety of medically important human gene products have been cloned. These include insulin, plasminogen activator, α 1 anti-trypsin, and coagulation factors. The present invention, however, encompasses the expression of any and all desired medically and/or biologically relevant gene products.

Once in the cell, the heterologous gene product is expressed in the tissue of choice at levels to produce functional gene products. The general consensus is that correct tissue-specific expression of most transfected genes is achievable. For correct tissue specificity, it may be important to remove all vector sequences used in the cloning of the DNA sequence of interest prior to introduction into the cell and formation of the artificial chromosome. Thus, the heterologous gene of interest can be incorporated into the artificial chromosome in a controlled manner so that the naturally-occurring sequences are present in their naturally-occurring configuration, and tissue specificity is assured.

Synthetic chromosomes can be introduced into human stem cells or bone marrow cells. Other applications will be clear to those of skill in the art.

A variety of ways have been developed to introduce vectors into cells in culture and into cells in tissues of an animal or human patient. Methods for introducing vectors into mammalian and other animal cells include calcium

phosphate transfection, the DEAE-dextran technique, micro-injection, liposome-mediated techniques, cationic lipid-based techniques, transfection using polybrene, protoplast fusion techniques, electroporation, and others. These techniques are well known to those of skill in the art, and are described in many readily available publications and been extensively reviewed. Some of the techniques are reviewed in *Transcription and Translation, A Practical Approach*, Hames, B.D. & Higgins, S.J., eds., IRL Press, Oxford (1984), herein incorporated by reference for their relevant teachings, and *Molecular Cloning*, 2nd Edition, Maniatis *et al.*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989), herein incorporated by reference for its relevant teaching.

In the description, reference has been made to various methodologies known to those of skill in the art of molecular biology. Publications and other materials setting forth such known methodologies to which reference is made are incorporated herein by reference for their relevant teachings.

A standard reference work setting forth the general principles of recombinant DNA technology is Maniatis, T. *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989).

Definitions

All terms pertaining to recombinant DNA technology are used in their art-recognized manner and would be evident to one of ordinary skill in the art.

The terms "Y alpha satellite" and "Y α " are used interchangeably and refer to alpha satellite DNA derived from the human Y chromosome.

The terms "17 alpha satellite" and "17 α " are used interchangeably and refer to alpha satellite DNA derived from human chromosome 17.

Alpha satellite DNA is a tandemly-repeated DNA sequence present at human centromeres and that comprises a basic monomeric repeat of

approximately 170 bps. This small repeat is organized into higher order units that have been shown to be specific to one or a small group of human chromosomes.

The term "centromeric" means that region of the chromosome that is constricted and is the site of attachment of the spindle during meiosis or mitosis. It is necessary for the proper segregation of chromosomes during meiosis and mitosis and is therefore an essential component of artificial chromosomes. Centromeric DNA comprises a DNA that directs or supports kinetochore formation and thereby enables proper chromosome segregation. Centromeric DNA at active, functional, centromeres is associated with CENP-E during mitosis, as demonstrated by immunofluorescence or immunoelectron microscopy. By "associated" is meant that the centromeric DNA and CENP-E co-localize by FISH and immunofluorescence.

"Essential chromosome functions" are discussed in the description and background above. These include mitotic stability without experimental selective pressure, substantially 1:1 segregation, autonomous replication, i.e., centromere, telomere, and origin of replication functions.

The term "functional equivalent" denotes a genetic function that arises from a different DNA or protein sequence, but which provides the same biological function.

The term "gene product" denotes a DNA, RNA, protein or peptide.

The term "genomic DNA" encompasses one or more cloned fragments or fragments from a restriction digest or other mixture of sequences and sizes, for example mechanically sheared DNA, or DNA synthesized *in vitro*. The DNA could be derived from the same chromosome (as, for example, when cloned fragments are used or when DNA from a purified chromosome is digested) or from different chromosomes (as, for example, when a genomic restriction digest is used for transfection).

The term "genomic" refers to DNA naturally found in the genome of an organism. However, the inventors also recognize that the function of this genomic DNA could be carried out by DNA from other sources, for example,

synthetic DNA that has a sequence not found in nature. Thus, as used herein, "genomic" DNA is also used generically to refer to the DNA that is introduced into a cell along with the centromeric (telomeric) DNA described herein, and which DNA expresses a gene product, or causes expression of a gene product, and allows the formation, in a cell, of a chromosome from purified DNA without the integration of the purified DNA into an endogenous chromosome in the cell. This DNA could thus be synthetic or derived from any organism and can be of any size as long as it contains the requisite expressible sequence and the function discussed above.

Therefore, in addition to the centromeric DNA, the artificial chromosome that is encompassed in the invention essentially contains DNA sequences that express a gene product, or causes expression of a gene product, and that allows the formation of a chromosome from purified DNA without the integration of the purified DNA into an endogenous chromosome in the cell. The sequence that functions to provide the chromosomal function (e.g., non-integration) and the expression sequence can be the same sequence. Thus, it is within the contemplation of the inventors that the expressible sequence also provides the other functions. Alternatively, the sequence that provides the chromosomal function and the expression function may be different sequences and from different sources.

In a specific disclosed embodiment, the genomic DNA is derived from a *Not I* restriction digest. Therefore, in a preferred embodiment, DNA that allows the formation of a chromosome from purified DNA without the integration of the purified DNA into an endogenous chromosome is derived from a restriction fragment generated by the digestion of total genomic DNA with a restriction enzyme having the recognition site (8 nucleotides) of *Not I*. However, it is well within the contemplation of the inventors to use restriction fragments and other fragments of naturally-occurring genomic DNA, that are smaller than those generated by *Not I* and comparable enzymes. For example, the inventors contemplate reducing the size of the DNA while retaining the functions above.

Therefore, in a highly preferred embodiment, the DNA is pared down to contain only the DNA necessary to provide for the expression of one or more genes of interest and to provide the function of allowing the formation of the chromosome from purified DNA without prior integration of the purified DNA into an endogenous chromosome.

The source of these DNAs need not be the same. Thus, the expression sequence can be derived from one organism and the sequence that provides the chromosomal function can be from another organism. Further, one or both sequences can be synthesized *in vitro* and need not correspond to naturally occurring sequences. In this respect, the sequences need not strictly be "genomic". The only restriction on the sequences is that they provide the functions indicated above.

The term "heterologous" denotes a DNA sequence not found in the naturally-occurring genome in which cell the artificial mammalian chromosome is introduced. Additionally, if the sequence is found, additional copies are considered "heterologous" because they are not found in that form in the naturally-occurring genome. As discussed above, the heterologous DNA can simultaneously be the desired expression sequence(s) and the "genomic DNA". "Expressible" DNA may not itself be expressed but may allow or cause the expression of another DNA sequence, heterologous or endogenous. This is the case if the DNA is regulatory, for example.

By "higher order repeat" is meant a repeating unit that is itself composed of smaller (monomeric) repeating units. The basic organizational unit of alpha satellite arrays is the approximately 171 bp alphoid monomer. Monomers are organized into chromosome-specific higher order repeating units, which are also tandemly repetitive. The number of constituent monomers in a given higher order repeat varies, from as little as two (for example, in human chromosome 1) to greater than 30 (human Y chromosome). Constituent monomers exhibit varying degrees of homology to one another, from approximately 60% to virtual

sequence identity. However, higher order repeats retain a high degree of homology throughout most of a given alphoid array.

The term "mammalian chromosome" means a DNA molecule or genetic unit that functions as a chromosome in a mammalian cell.

5 The term "naked DNA" means DNA that is unassociated with any of the biological (chromosomal or cellular) components with which it is normally associated in a naturally-occurring chromosome, for example histones, non-histone chromosomal proteins, RNA, transcription factors, topoisomerases, scaffold proteins, centromere-binding proteins, and telomere-binding proteins.
10 Such DNA can be isolated from cells and purified from the non-DNA chromosomal components. Alternatively, this DNA can be synthesized *in vitro*.

The term "naturally-occurring" denotes events that occur in nature and are not experimentally-induced.

An origin of replication indicates a site of initiation of DNA synthesis.

15 The term "isolated" refers to DNA that has been removed from a cell. The term "purified" refers to isolated DNA that has been substantially completely separated from non-DNA components of a cell or to DNA that has been synthesized *in vitro* and separated substantially completely from the materials used for synthesis that would interfere with the construction of the chromosome
20 from the DNA. A purified DNA can also be a DNA sequence isolated from the DNA sequences with which it is naturally associated.

A replicon is a segment of a genome in which DNA is replicated and by definition contains an origin of replication.

25 The phrase "retains all the functions of a natural mammalian chromosome" means that the chromosome is stably maintained in dividing mammalian cells as a non-integrated construct, without experimental selective pressure, indicating at least centromeric, telomeric (for linear chromosomes), origin of replication functions, and gene expression.

30 The term "mitotically stable" denotes that the synthetic or artificial chromosome remains present in at least 50% of the cells after ten generations in

the absence of experimental selective pressure (such as drug selection and the like), and most preferably, that after 30 generations, it is present in at least 10% of the cells; and preferably, the synthetic chromosome exhibits 1:1 segregation greater than 99% of the time.

5 By "stably transformed" is meant that the cloned DNA array containing the repeating units is capable of being propagated in the micro-organism host cell for at least 50 generations of growth with a recombination frequency of less than 0.6 % per generation (for 174 kb arrays) and a recombination frequency of less than 0.2 % (for 130 kb arrays). Arrays smaller than 130 kb exhibit little or no
10 recombination when cloned by the method of the invention.

 The terms "synthetic" or "artificial" are used interchangeably. A "synthetic" or "artificial chromosome" is a construct that has essential chromosome functions but which is not naturally-occurring. It has been created by introducing purified DNA into a cell. Since the chromosome is composed
15 entirely of transfected DNA, it is referred to as synthetic or artificial. An artificial or synthetic chromosome is found in a configuration that is not naturally-occurring.

 The term "transfecting" denotes the introduction of nucleic acids into a cell. The nucleic acid thus introduced is not naturally in the cell in the sequence
20 introduced, the physical configuration, or the copy number.

 A telomere denotes the end of a chromosome comprising simple repeat DNA that is synthesized by a ribonucleoprotein enzyme called telomerase. The function is to allow the ends of a linear DNA molecule to be replicated.

 A nucleic acid molecule such as a DNA or gene expresses a polypeptide
25 or gene product if the molecule contains the sequences that code for the polypeptide and the expression control sequences which, in the appropriate host environment, provide the ability to transcribe, process and translate the genetic information contained in the DNA in a protein product and if such expression control sequences are operably linked to the nucleotide sequence which encodes
30 the polypeptide. However, as discussed herein, a gene product need not be

restricted to a polypeptide gene product but may encompass RNA. Further, genetic defects that are capable of being corrected by the artificial mammalian chromosomes when used as expression vectors may be defects that operate *in cis* to effect further gene expression.

5 An operable linkage is a linkage in which the regulatory DNA sequences and the DNA sequence sought to be expressed are connected in such a way as to permit gene expression. The precise nature of the regulatory regions needed for gene expression may vary from organism to organism but in general include a promoter region, 5' non-coding sequences involved with initiation of
10 transcription and translation such as the TATA Box, CAP Sequence, CAAT Sequence, and the like. If desired, the non-coding region 3' to the gene sequence coding for the protein may be obtained by the above-described methods. This region may be retained for its transcriptional termination regulatory sequences such as termination and polyadenylation. Thus, by retaining the 3' region
15 naturally contiguous to the DNA sequence coding for the protein, the transcription termination signals may be provided. Where the transcriptional termination signals are not satisfactorily functional in the expression host cell, then a 3' region functional in the host cell may be substituted.

20 The following examples do not limit the invention to the particular embodiments described, but are presented to particularly describe certain ways in which the invention may be practiced.

Examples

Example 1

Construction of Chromosome 17 Alpha Satellite Vectors

25 In order to clone and propagate alpha satellite DNA in *E. coli* using BAC vectors, a series of tandem alpha satellite arrays of various sizes were constructed.

The structure of the higher order alpha satellite repeat from human chromosome 17 has been described previously (Waye and Willard, *Mol. Cell. Biol.* 6(9):3156-3165 (1986)). The predominant higher order repeat of human chromosome 17 is 2.7 kb in length, and consists of 16 alphoid monomers flanked by *EcoR* I sites. A discrete structural unit of alpha satellite DNA, the higher order repeat, derived from human chromosome 17 or the human Y chromosome was cloned into the plasmid cloning vector pACYC184 (New England Biolabs) by digesting human genomic DNA with the restriction enzyme *EcoR* I. The nucleotide sequence of the cloned higher order repeats was verified by DNA sequence analysis.

Polymerase chain reaction (PCR) was used to amplify a single 2.7 kb higher order repeat monomer unit such that complementary restriction sites (*Bam*H I and *Bgl* II) were created at opposing ends of the higher order register. The precise length of the higher order repeat was maintained. The primer pair used to amplify this fragment was

VB100 - 5' ...gggcgggagatctcagaaaattcttgggatgattgagttg (SEQ ID NO.:2)

and

VB101 - 5' ...gggcgggatcccttctgtcttcttttataggaagttatt (SEQ ID NO.:3).

The modified higher order repeat was cloned into the BAC vector pBAC108L (a gift from Bruce Birren, California Institute of Technology, Pasadena, CA) by digesting the amplified fragment with *Bam*H I, gel purifying the insert DNA, and ligating into vector DNA which had been digested with *Bam*H I and *Hpa* I and gel purified. The resulting plasmid was designated pBAC-17 α l.

To construct a synthetic dimer of alpha satellite DNA, aliquots of pBAC-17 α l were digested separately with either: (1) *Bam*H I + *Sfi* I, or (2) *Bgl* II + *Sfi* I. Following gel electrophoresis, the 2.7 kb alpha satellite band from the *Bgl* II/*Sfi* I digest was excised and ligated to the excised pBAC-17 α l *Bam*H I/*Sfi* I fragment. Since *Bgl* II and *Bam*H I generate compatible overhangs, and *Sfi* I generates an asymmetric overhang that can only religate in a particular

orientation, the 2.7 kb fragment ligates to the vector DNA to create a tandem dimer arrayed in head to tail fashion. Ligation products were transformed into the bacterial strain DH10B by electroporation. Clones were analyzed by restriction analysis, and those that contained a tandemly arrayed modified dimer of the chromosome 17 alpha satellite were designated pBAC-17 α 2 (Figure 1). This strategy was repeated to create extended alpha satellite arrays consisting of 4, 8, 16, 32, 48, or 64 higher order alpha satellite repeats. A similar strategy was utilized to construct synthetic arrays of higher order repeats from other human chromosomes, such as the Y chromosome.

The construction of BAC vector containing 174 kb of alpha satellite DNA represents the largest amount of this class of DNA to be cloned and propagated in *E. coli* to date. Previous experiments have successfully cloned approximately 40 kb in *E. coli* using cosmids (Willard *et al.*, *Prog. Clin. Biol. Res.* 318:9-18 (1989)). Others have used medium copy number plasmids to clone arrays ranging in size from the about 171 bp alphoid monomer to 40 kb (Waye and Willard, *Nucleic Acids Res.* 15(18):7549-69 (1987)). In the studies reported in the art, a high frequency of recombination during propagation in *E. coli* was observed in the plasmids that contained the largest alphoid arrays. In contrast, during the propagation of the pBAC-17 α 64, little evidence of recombination products was observed, utilizing standard methods of plasmid purification. Since structural instability of alpha satellite DNA in microorganisms has been shown in the context of these cloning vectors, the preparations of the pBAC-17 α 64 were analyzed for the presence of obviously rearranged arrays utilizing gel electrophoresis. By this assay, the presence of significant levels of rearranged plasmid was not detectable.

Example 2

Assay for Stability of Cloned Repetitive DNA in E. coli

Although no evidence for high levels of recombination and/or deletion in the synthetic alpha satellite arrays from Example 1 was observed following propogation in *E. coli*, it was possible that recombination was occurring at relatively high levels, but the large number of different deletion products prevented any one product from being detectable. Therefore, the rate of recombination of these constructs utilizing a highly sensitive assay was determined. In addition, because larger arrays might be expected to be less stable than smaller ones, several different size constructs were examined. The stability of these constructs was examined below.

Stability assays were carried out using three different alpha satellite array sizes which had been cloned into pBAC108L. These constructs, pBAC-17 α 32, pBAC-17 α 48, and pBAC-17 α 64, contain 87 kb, 130 kb, and 174 kb of alpha satellite DNA, respectively. Following transformation (electroporation) into the *E. coli* strain, DH10B (GIBCO BRL), single clones were picked and analyzed. Because it is possible that the transformation process itself may lead to DNA rearrangements, only clones containing predominantly full-length constructs, as judged by restriction digest and electrophoresis, were saved as glycerol stocks.

To begin the stability assay, *E. coli* cells from a glycerol stock of each construct were streaked onto LB plates containing 12.5 μ g/ml chloramphenicol. Eight of the resulting colonies were picked and grown individually to saturation in 5 ml of LB containing 12.5 μ g/ml chloramphenicol (approximately 20 generations). The plasmid DNA from each clone was then purified, digested with *Bam*H I, and separated by pulse field gel electrophoresis. Clones that contained any full length plasmid were said to have full length plasmid at the single cell stage. Clones that did not contain any detectable full-length plasmid were said to be the result of a recombination event prior to restreaking (i.e., a rearrangement

that occurred during production of the glycerol stock) and were excluded. Of the clones that contained some full-length plasmid, 10 cultures were picked at random, diluted 1 to one million into fresh LB containing 12.5 µg/ml chloramphenicol, and growth to saturation (approximately 30 generations). From a single cell to this final saturated culture, approximately 50 generations of growth occurred.

In order to determine the percent of plasmids that rearranged during these 50 generations, each saturated culture was streaked onto LB plates containing 12.5 µg/ml chloramphenicol. Individual colonies were then grown to saturation in 1.5 ml LB containing 12.5 µg/ml chloramphenicol. Following growth, the DNA was purified and analyzed by restriction digest (*Bam*H I) and PFGE. Any clone that contained detectable full length plasmid was scored as unrearranged during the 50 generation experiment. Conversely, any clone which did not contain any detectable full-length plasmid was scored as rearranged during the 50 generation experiment. To calculate the average rearrangement frequency per generation for each construct, the fraction of rearranged clones was determined after 50 generations. One minus this value is equal to the fraction of unrearranged clones (after 50 generations). The fraction of clones that rearrange after one generation is 1 minus the 50th root of the fraction of unrearranged clones after fifty generations. This is summarized in the following equation:

$$X = 1 - (1 - Y)^{1/50}$$

where X is the fraction of clones which rearrange per generation and Y is the fraction of rearranged clones after 50 generations of growth.

Using this strategy, the recombination frequency of three alpha satellite containing constructs was determined. After 50 generations of growth, 0% (n=9) of the pBAC-17α32 clones recombined to truncated forms. Recombinants were detected for the pBAC-17α48 and pBAC-17α64 at a level of 8.5 % (n=59) and 25% (n=84), respectively. This corresponds to a per generation recombination frequency of 0.18% for pBAC-17α48 and 0.57% for pBAC-17α64. Thus, this recombination frequency is significantly lower than that reported for other

cloning vectors containing far less alphoid DNA (for example, 40 kb) and which are grown for less generations (for example, 30 generations).

The results show that alpha satellite arrays up to at least 174 kb in size can be stably propagated in *E. coli* using BAC vectors in the methods of the invention. The 174 kb and 130 kb arrays recombine at a frequency of 0.57 % and 0.18% per generation, respectively. Thus, using pBAC-17 α 64 as an example, following 50 generations of growth from a single cell, approximately 1,000 titers of saturated bacterial culture can be produced from a single cell and at least 75% of the cells will contain full-length pBAC-17 α 64, on average. This degree of rearrangement falls within the expected acceptable range for the large scale production of alpha satellite-containing human artificial chromosomes for use in gene therapy.

In addition to determining the frequency of alpha satellite DNA rearrangement in pBAC108L, a correlation between the size of a highly repetitive alpha satellite array and its stability in this vector was established. Based on the recombination frequencies determined above, the minimum upper size limit estimate of homogeneous alpha satellite DNA in BAC vectors (assuming 50% full length clones after 50 generations to be acceptable) is conservatively estimated to be between 200 and 215 kb (Figure 2). This was determined by extrapolation using the computer program Cricket Graph. This is a minimum estimate of alphoid capacity, as other lines are found that fit the data and produce larger estimates than those stated above. From this correlation, it is estimated that, when using 200-215 kb arrays, and propagation in bacterial strain DB10B, greater than 50% of the plasmid will be full length after 50 generations. It is likely that this upper size figure could be extended by utilizing the BAC vector in conjunction with specialized recombination-defective bacterial strains. Furthermore, this estimate is based on maximally homogeneous arrays. The stability of diverged arrays including, certain natural alpha satellite arrays, should exceed this estimate.

The experiment described here represents the first stable cloning and propagation of an alpha satellite array larger than 50 kb in *E. coli*. Previously, alpha satellite DNA has been cloned in *E. coli* using cosmids (Haaf *et al.*, *Cell* 70(4):681-96 (1992). In addition to the relatively small size of these arrays (equal to or less than 40 kb), the integrity and stability of these arrays was not analyzed. Alpha satellite DNA has also been cloned using YACs (Neil *et al.*, *Nucleic Acids Res.* 18(6):1421-8 (1990)). In these studies, the instability of the alpha satellite arrays was noted, and additional manipulations, such as agarose gel purification, were required to obtain preparations containing predominantly full-length arrays. In addition, there are certain disadvantages to using YACs to propagate alpha satellite DNA. Perhaps the most important of these relates to the topology of YACs. In general, YACs are linear DNA molecules, and therefore, simple alkaline lysis purification methodology can not be used to purify the alpha satellite construct away from contaminating yeast chromosomes. Instead, pulsed field gel electrophoresis, a separation method which is not amenable to scale-up, must be used. Finally, the linear topology of YACs renders them particularly susceptible to shearing during purification. Here, it has been demonstrated that the alpha satellite-containing BACs can be harvested and purified away from *E. coli* chromosomal DNA without substantial shearing.

Previous studies suggest that alpha satellite DNA is an important component of the functional human centromere. Naturally-occurring alpha satellite arrays range in size from 230 kb to several megabases in length (Oakey and Tyler, *Genomics* 7(3):325-30 (1990)); (Wevrick and Willard, *Proc. Natl. Acad. Sci. USA* 86(23):9394-8 (1989)). However, recent studies suggest that as little as 140 kb of alpha satellite DNA is sufficient to confer centromere function in human cells (Brown *et al.*, *Human Molecular Genetics* 3(8):1227-1237 (1994)). The alpha satellite array constructs described herein are the first that allow the large scale, stable production of alpha satellite arrays which are large enough to satisfy the alpha satellite requirements of a functional human

centromere. These constructs can serve as the backbone of synthetic human chromosomes.

Example 3

Construction of Y Chromosome Alpha Satellite Vectors

5 Construction was as in Example 1, except that DNA from the human male Y chromosome cell line GM07033 was used; DNA from any normal human male cell line would be equivalent. The predominant higher order alphoid repeat on the human Y chromosome is 5.7 kb in length, and is demarcated by flanking *EcoR* I sites. The 5.7 kb higher order repeat from the Y chromosome alphoid
10 array was cloned into a standard *E. coli* cloning vector, pACYC184 (New England Biolabs). The ends of the higher order repeat were then modified using PCR to create a *BamH* I site at one end and a *Bgl* II site at the other, replacing the existing *EcoR* I site. The modified higher order repeats were cloned into the pBAC108L cloning vector as above.

15

Example 4

Construction of Hybrid Alpha Satellite Vectors

Construction was as in Example 1, except that alphoid DNA from both human chromosome 17 and the human Y chromosome was used. Two types of arrays were constructed. One array was a simple alternating repeat wherein one
20 higher order repeat unit of chromosome 17 alphoid DNA alternated with one higher order repeat unit from the Y chromosome alphoid DNA. The second type of array that was constructed alternated a dimeric unit of the chromosome 17 higher order repeat of alphoid DNA with one unit of the chromosome Y repeat of alphoid DNA. In each case, as with the above examples, the proper phasing
25 of the higher order repeats derived from each chromosome was retained at the junction of the synthetic hybrid.

Example 5

Construction of Artificial Mammalian Chromosome

Experimental Procedures

Description of DNA constructs

5 Standard molecular biology techniques were used to construct all plasmids described here (Sambrook, J. *et al.*, eds., "Molecular Cloning", Cold Spring Harbor Laboratory Press (1989)). Cloning of the alpha satellite higher order repeat from the Y chromosome and chromosome 17 has been described previously (Wolfe, J. *et al.*, *J. Mol. Biol.* 182:477-485 (1985); Van Bokkelen, G.B. *et al.*, "Method for Stably Cloning Large Repeating Units of DNA", U.S. Patent Application(1995)); Waye & Willard, *Mol. Cell. Biol.* 6:3156-65 (1986)).
10 By directional cloning through the creation of the appropriate restriction sites, successively larger alpha satellite arrays have been created in the plasmid pBAC108L Van Bokkelen, G.B. *et al.*, "Method for Stably Cloning Large Repeating Units of DNA", U.S. Patent Application No. 08/487,989, filed June 7, 1995, which is incorporated herein by reference for teaching the cloning of large tandem arrays of repetitive sequences.

Plasmids used in the experiments

20 pBAC108L has been described previously (Shizuya, H. *et al.*, *Proc. Natl. Acad. Sci. USA* 89:8794-7 (1992)). pVJ105 is a modified version of pBAC108L that contains additional restriction sites in the polylinker and a β -geo expression unit consisting of the CMV immediate early gene promoter and SV40 polyadenylation signal (Seed, B., *Nature* 329:840-2 (1987); Seed & Aruffo, *Proc. Natl. Acad. Sci. USA* 84:3365-9 (1987)), the β -geo open reading frame

(MacGregor, G.R. *et al.*, *Development* 121:1487-96 (1995)), and the UMS transcriptional termination sequence (Heard, J.M. *et al.*, *Mol. Cell. Biol.* 7:2425-34 (1987); McGeady, M.L. *et al.*, *DNA* 5:289-98 (1986); Salier & Kurachi, *Biotechniques* 7:30-1 (1989)). pBACY α 16 (92 kb of Y alpha satellite) consists of 16 identical higher order repeats cloned head-to-tail into pBAC108L. pBAC17 α 32 (87 kb of 17 alpha satellite) consists of 32 identical higher order repeats cloned head-to-tail into pBAC108L. pVJ105-Y α 16 was made by cloning the alpha satellite array from pBAC108L into pVJ105. Following linearization with *Bam*HI and *Sfi*I, the direction of β -geo expression is toward the alpha satellite array. pVJ105-17 α 32 was made by cloning the alpha satellite array from pBAC17 α 32 into pVJ105. Following linearization as above, the direction of β -geo transcription is toward the alpha satellite array. All plasmids were purified by alkaline lysis (Sambrook, J. *et al.*, eds., "Molecular Cloning", Cold Spring Harbor Laboratory Press (1989)) followed by agarose gel purification.

Creation of alpha satellite arrays >100 kb by multimerization

To create Y alpha satellite arrays, pVJ105 Y α 16 was digested with *Bam*HI and *Sfi*I and gel purified by pulsed field gel electrophoresis (PFGE). Additional alpha satellite DNA was prepared by digesting pBACY α 16 with *Bam*HI and *Bgl*II and gel purifying the 92 kb alpha satellite fragment by PFGE as above. Following band isolation, the agarose bands were equilibrated in 10 mM Tris pH 7.5, 100mM NaCl, 10 mM MgCl₂ and then melted at 65° for 5 minutes. The two fragments were then combined at a molar ratio of 5:1 (pBACY α 16 alpha satellite fragment: pVJ105-Y α 16 fragment). ATP (1 mM final) and T4 Ligase (5 units) were added and the reaction was incubated at 37 °C for 4 hours in the presence of *Bam*HI (40 units) and *Bgl*II (40 units). β agarase (3 units) was added and the reaction was incubated at 37°C for 1 hour. The reaction was then placed on ice for 1 hour prior to transfection into HT1080 cells. To create extended alpha satellite arrays for 17 alpha satellite DNA, the above procedure was used with

pVJ105-17 α 32 and pBAC17 α 32 in place of pVJ105-Y α 16 and pBAC17 α 32, respectively.

Preparation of high molecular weight human genomic DNA

Four 150 mm plates containing HT1080 cells were grown to confluency, removed from the plates with trypsin/EDTA, and washed with 100 ml PBS. High molecular weight DNA was harvested in low gelling temperature agarose plugs (Sambrook, J. *et al.*, eds., "Molecular Cloning", Cold Spring Harbor Laboratory Press (1989)). Approximately 1 μ g of human genomic DNA was digested with *NotI*. Following digestion, *NotI* was inactivated by heating the reaction to 70°C for 5 min. Prior to transfection, the agarose plug was digested with 3 units of β -agarase.

Preparation of telomeric DNA

Human telomeric DNA was generated by PCR using primers 42a (5'GGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGG3'; SEQ ID NO.:4) and 42b (5'CCCTAACCCTAACCCTAACCCTAACCCTAACCCTAACCCTAACC3'; SEQ ID NO.:5) (Ijdo, J.W. *et al.*, *Nucleic Acids Res.* 19:4780 (1991)). Each PCR reaction contained 250 ng of 42a and 42b, 5 Units Taq polymerase, 250 μ M dNTPs, 3.3 mM MgCl₂ in 1X PCR Buffer (Gibco BRL). The PCR reaction was carried out for 35 cycles in a Perkin Elmer 9600 Thermal cycler using the following temperature profile: 95°C for 20 seconds, 40°C for 20 seconds, 72°C for 2 minutes. Following PCR, each reaction was subjected to agarose gel electrophoresis to purify telomeric DNA that is greater than 1 kb in size. This DNA was excised from the gel and purified away from the agarose using Magic Prep columns according to the manufacturer's instructions (Promega, WI).

Transfection of human cells

Prior to transfection, pVJ105 Y α 16 and pVJ105 17 α 32 were digested with *Bam*HI and *Sfi*I; pBac Y α 16 and pBac Y α 32 were digested with *Bam*HI and *Bgl*II. The DNA was then purified by PFGE, equilibrated against 10 mM Tris pH 7.5, 100 mM NaCl, and combined with telomeric DNA and/or *Not*I digested human genomic DNA. In some cases, the alpha satellite arrays were extended using the directional ligation approach described in Figure 3.

The DNA components for each transfection were combined and gently mixed. Transfections contained either pVJ105 Y α 16 (0.5-1 μ g), pVJ105 17 α 32 (0.5-1 μ g), or pVJ105 VK75 (0.5-1 μ g). Where indicated, the transfections also contained purified Y α 16 arrays (0.5-1 μ g), 17 α 32 arrays (0.5-1 μ g) telomeric DNA (75-250 ng), human genomic DNA (1-3 μ g) and/or VK75 fragment (0.5-2 μ g). 1 ml serum free α -MEM media (MediaTech) was added. 7.5 μ l lipofectin was then added, and the solution was incubated at room temperature for 5 minutes. The DNA:lipofectin mixture was added to 2×10^6 HT1080 cells, according to the manufacturer's instructions (Gibco BRL). After a 16 hour incubation at 37°C, the DNA:lipofectin solution was removed and complete media was added to the cells. At 36 hours post transfection, the cells were removed from the wells with trypsin/EDTA and transferred to a 100 mm plate containing complete media supplemented with 300 μ g/ml G418. On the seventh day of selection, the media was replaced with fresh complete media supplemented with 300 μ g/ml G418. After 12 days of selection, individual colonies were isolated using sterile cloning rings and placed into 24 well plates. The individual clones from each transfection were then expanded under selection into 100 mm plates. A portion of each culture was frozen for future analysis, while the bulk was harvested for analysis by FISH.

Cell culture

HT1080 cells were grown in Alpha MEM media (Gibco/BRL, Bethesda, MD) supplemented with 15% fetal bovine serum (Hyclone), penicillin/streptomycin, and glutamine. The subclone of HT1080 used in these experiments was tetraploid.

Plate staining

Cells containing the synthetic chromosomes were plated in 6 well plates at 10% confluency. Untransfected HT1080 cells were similarly plated and used as a negative control. When the cells reached 70 % confluency, the media was removed and the cells were washed with 2 ml PBS. After removing the PBS, 1 ml of fix solution (2% formaldehyde, 0.2% glutaraldehyde in PBS) was added to each well and the plate was incubated at room temperature for 4 minutes. The fix solution was removed and the cells were immediately washed with 2 ml PBS. Finally, PBS wash was removed and 1 ml of staining solution (5mM potassium ferricyanide, 5mM potassium ferrocyanide, 5 mM MgCl₂, and 1 µg/µl X-Gal in PBS) was added to each well and the plate was incubated for 12 hours at 37°C. The cells were washed with PBS and imaged using a light microscope and associated imaging hardware (Oncor, Gaithersburg, MD).

Fluorescence in situ hybridization

HT1080 cells were grown on 100 mm tissue culture plates, harvested for FISH, and mounted onto slides according to published procedures (Verma, R. & Babu, A., *Human Chromosomes, Principles and Techniques*, 2nd Edition, McGraw-Hill, Inc. (1995)). To detect alpha satellite sequences on the synthetic chromosomes and on the endogenous chromosomes, chromosome specific alpha

satellite probes were used according to manufacturers instructions (Oncor, Gaithersburg, MD).

Determination of synthetic Y alpha satellite DNA content in clones containing synthetic chromosomes

5 Genomic DNA was harvested from HT1080 cells and from clones 22-6, 22-7, 22-11, 22-13, and 23-1 according to published procedures (Sambrook, J. *et al.*, eds., *Molecular Cloning*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989)). Approximately 10 µg DNA from each clone was then digested with *EcoRI* (50 units) and *PstI* (50 units) overnight at 37°C. The
10 samples were then electrophoresed through a 0.8% agarose gel, transferred to Nytran membrane, and hybridized to a 1 kb Y alpha satellite probe in 25% formamide/10% dextran/0.5% SDS/0.5M NaCl/200 µg/ml salmon sperm DNA overnight at 65°C. *EcoRI* and *PstI* both cleave once in the endogenous Y alpha satellite higher order repeat to give a 4 kb and a 1.7 kb band. However, due to
15 the method used to create the synthetic Y alpha satellite arrays, *EcoRI* does not cleave the synthetic higher order repeat. As a result, *EcoRI* and *PstI* digestion of the synthetic array results in a 5.7 kb band. Since we know that the endogenous Y alpha satellite array is 1 mb in length, we can determine the amount of synthetic alpha satellite DNA in the cells containing synthetic chromosomes by
20 determining the ratio of the 5.7 kb band (synthetic array) to the 4.0 kb band (endogenous array). It is not necessary to consider the 1.7 kb band since it does not hybridize with the probe under these hybridization conditions. It is important, however, to consider that most of these clones contain 2 Y chromosomes (because they are tetraploid) and only a single synthetic chromosome. One
25 exception to this is clone 22-13 which contains a single Y chromosome and appears to be diploid. Thus, for clones 22-6, 22-7, and 22-11, the amount of synthetic alpha satellite DNA per cell is estimated by the following equation:

-55-

$$\frac{\text{Intensity of 5.7 kb band}}{\text{Intensity of 4 kb band}} \times 1 \text{ mb} \times 2$$

For clone 22-13, the following equation was used:

$$\frac{\text{Intensity of 5.7 kb band}}{\text{Intensity of 4 kb band}} \times 1 \text{ mb}$$

Immunofluorescence

Anti-CENP immunofluorescence was carried out according to published procedures (Sullivan & Schwartz, *Hum. Mol. Genet.* 4:2189-2197 (1995)).

5 Briefly, HT1080 cells were grown in tissue culture plates until approximately 80% confluency. Colcemid was then added to a final concentration of 40 ng/ml and the cells were incubated at 37°C for 75 minutes. The media was carefully removed and the cells were released from the plate by incubation with trypsin/EDTA for 3 to 5 minutes. To neutralize the trypsin, complete media was

10 added to the cells and the resulting cell suspension was counted using a hemocytometer and spun at 1000 rpm in a Jouan CT422 centrifuge. The supernatant was discarded and the cells were resuspended at 0.6×10^5 cells/ml by slowly adding hypotonic solution (25 mM KCl, 0.27% sodium citrate). Cells were incubated in hypotonic solution for 12 minutes at room temperature. 500

15 μ l of cells were then added to a cytofunnel and spun at 1900 rpm for 10 minutes in a Shandon Cytospin 3 centrifuge. The slides were then incubated in 10 mM Tris pH 7.7, 120 mM KCl, 20 mM NaCl, 0.1% Triton X-100 for 12 minutes. Diluted antibody (50 μ l, 1/1000 in 1 mM triethanolamine, 25 mM NaCl, 0.2 mM EDTA, 0.5% Triton X-100, 0.1% BSA) was added to each slide and a plastic

20 cover slip was positioned over the cells. Following a 30 minute incubation at 37°C, the coverslip was removed, and the slides were washed 3 x 2 minutes in a

Coplin jar containing KB (10 mM Tris pH 7.7, 150 mM NaCl, 0.1% BSA). FITC-labeled anti-rabbit Ig was added (50 μ l of 1/100 in KB) and a plastic cover slip was placed over the cells. Following a 30 minute incubation at 37 °C, the slides were washed 3 x 2 minutes in a Coplin jar containing KB. Before viewing,
5 the slides were counterstained with 10 μ l DAPI (2 μ g/ml in antifade). Images were collected using a fluorescent microscope and imaging system (Oncor, Gaithersburg, MD).

Mitotic stability time course

Following cloning, cells were expanded into two 100 mm plates and
10 grown in the presence of 300 μ g/ml G418. At 80% confluency, one plate for each clone was harvested for FISH analysis using the protocol described above. These cells serve as the time zero point of the time course. The other plate was split 1/16 into a 100 mm plate and grown to confluency in complete media lacking G418. As soon as the culture reached confluency, the cells were split
15 1/16 and grown in complete media lacking G418. This process was repeated for the period of time indicated in Table 2. At various time points, a portion of the culture was harvested for FISH and analyzed for the presence of the transfected alpha satellite (17 α or Y α). For each intact chromosome spread, the number of Y alpha satellite (or 17 alpha satellite) signals and their chromosomal positions
20 were determined.

Results

The mammalian centromere is a complex chromosomal element thought to consist of large blocks of repetitive DNA, called alpha satellite. One of the major impediments inhibiting the elucidation of mammalian centromere structure
25 and preventing the development of artificial human chromosomes has been the inability to clone large segments of this class of DNA. Recently, methods for the

cloning and large scale production of alpha satellite DNA up to approximately 175 kb in length have been developed (Van Bokkelen, G.B. *et al.*, entitled "Method for Stably Cloning Large Repeating Units of DNA" U.S. Appl. No. 08/487,989, filed June 7, 1995. Equally important, the use of a directional cloning strategy allows the creation of alpha satellite arrays of known composition and structure.

In order to facilitate the formation of a functional centromere from naked transfected alpha satellite DNA, the inventors hypothesized that it could be advantageous to transfect alpha satellite DNA which is greater than 175 kb in size. Previously, the largest contiguous alpha satellite array to be transfected into mammalian cells was 120 kb (Larin, Z. *et al.*, *Hum. Mol. Genet.* 3:689-95 (1994)). To produce alpha satellite DNA much larger than 175 kb, the directional ligation strategy shown in Figure 3A was used. This *in vitro* technique allows the production of contiguous, uninterrupted Y alpha satellite arrays up to 736 kb in length (Figure 3B, lanes 2-4). As a control, VK75 (a 75 kb *BssHII* fragment) was ligated in the presence and absence of *BssHII* (Figure 3B, lanes 5-7). Since *BssHII* ends regenerate a *BssHII* site when ligated, the ladder of multimers is digested down to constituent monomers when *BssHII* is included in the ligation reaction. Similar results were obtained in experiments carried out using *BamHI* fragments or *BglII* fragments (data not shown). This demonstrates that the recleavage reaction is efficient and that the ladder in lanes 2 and 3 are the result of head-to-tail ligations. Finally, to test for biological differences between separate families of alpha satellite DNA, extended arrays were also built consisting of alpha satellite DNA derived from chromosome 17 (data not shown).

The inventors have utilized these large purified alpha satellite arrays to produce synthetic chromosomes using the strategy outlined in Figure 4 and described in Experimental Procedures. By cotransfecting each of these chromosome components, the inventors reasoned that the cell would combine these elements to form a functional chromosome. Accordingly, HT1080 cells were transfected with various combinations of alpha satellite DNA, telomeric

DNA, and human genomic DNA. Following transfection, the cultures were placed under G418 selection for 10-14 days. Individual colonies were then isolated, expanded under selection, and harvested for FISH analysis.

Characterization of stable transfectants

5 As shown in Table 1, in clones from the majority of transfections, alpha satellite DNA had integrated into an endogenous chromosome. In many transfections that included telomeric DNA, a high incidence of alpha satellite integration events associated with chromosome truncations was observed also. It has been observed previously that telomeric DNA can be used to efficiently
10 truncate human chromosomes following integration (Barnett, M.A. *et al.*, *Nucleic Acids Res.* 21:27-36 (1993); Brown, K.E. *et al.*, *Hum. Mol. Genet.* 3:1227-37 (1994); Farr, C.J. *et al.*, *EMBO J.* 14:5444-54 (1995)). Here, telomeric DNA apparently integrated into the endogenous chromosome along with alpha satellite DNA and caused a truncation event.

15 In cells from a subset of transfections, however, synthetic chromosomes that contained the transfected alpha satellite DNA were observed (Table 2, transfections 22 and 23 and Figures 5-7). These positive transfections differed from the other transfections in two ways. First, prior to transfection, the alpha satellite DNA was preligated *in vitro* in the presence of *Bam*HI and *Bgl*II
20 (Figures 3A and 3B). This resulted in the generation of large, directional alpha satellite arrays ranging in size from 100 kb to 736 kb in length. Second, *Not*I digested human genomic DNA was included in the transfection. By including these two components, the essential DNA sequences necessary for synthetic chromosome formation were provided.

25 FISH analysis of clones from transfections 22 and 23 revealed that approximately 50% of the G418 resistant clones contained synthetic chromosomes (Table 1). In four of the five synthetic chromosome containing clones from these two transfections, the transfected alpha satellite DNA was

detectable only on the synthetic chromosome (Figures 4-6). That is, in the case of transfected Y alpha satellite DNA, only the synthetic chromosome and the Y chromosome had detectable signals by FISH. Likewise, in the case of transfected 17 alpha satellite DNA, only the synthetic chromosome and chromosome 17 had detectable signals for 17 α by FISH. Interestingly, synthetic chromosomes formed in both transfections 22 and 23. This demonstrates that alpha satellites from the Y chromosome and from chromosome 17 are both capable of facilitating synthetic chromosome formation. As further evidence that alpha satellite DNA is an important component of the synthetic chromosomes, the alpha satellite FISH signal encompasses most or all of each synthetic chromosome.

In cells that contain a synthetic chromosome, there were only two exceptions where alpha satellite DNA (derived from the same chromosome as the synthetic α satellite DNA used in the transfection) was detected on a chromosome other than the synthetic chromosome and Y chromosome (or chromosome 17 in cases where 17 α satellite was transfected). First, in clone 17-15, 17 alpha satellite DNA was detected on the synthetic chromosome, chromosome 17, and at the end of a C group chromosome (Figures 7A and B). Interestingly, this transfection contained unligated alpha satellite and telomeric DNA, but no human genomic DNA. One possibility is that the transfected DNA integrated into the endogenous chromosome, amplified, and broke back out. It is important to note that if non-alphoid, non-telomeric human sequences are necessary for chromosome function, then this mechanism of synthetic chromosome formation might be necessary to provide additional DNA elements in the absence of human genomic DNA in the transfection. In the one case in which a synthetic chromosome formed in the absence of cotransfected human genomic DNA, alpha satellite was also found integrated into an endogenous chromosome. This shows that genomic DNA is necessary for some aspect of synthetic chromosome formation or maintenance. Second, in clone 22-11, both a synthetic chromosome and a Y:14 chromosome translocation were observed (Figures 7C and D). By pulsed field gel electrophoresis, the inventors have demonstrated that the Y:14

translocation contains endogenous Y alpha satellite DNA, and not synthetic Y alpha satellite DNA. Thus, the synthetic alpha satellite DNA is only detectable on the microchromosome, and not on any endogenous chromosome.

Estimation of synthetic chromosome size

5 The amount of alpha satellite DNA present in the synthetic chromosome containing cells ranges from about 350 kb to 2 mb (Figure 8). This was determined by taking advantage of restriction site polymorphisms between the synthetic and endogenous alpha satellite arrays. By comparing the intensity of the synthetic alpha satellite band to the endogenous alpha satellite band on a
10 Southern blot, the ratio of synthetic alpha satellite DNA to endogenous alpha satellite DNA can be determined. Since the endogenous alpha satellite array is 1 mb in length (Larin *et al.*, *Hum. Mol. Genet.* 3:689-95 (1994)), and since the copy number of the Y chromosome (2 for clones 22-6, 22-7, and 22-11 and one for clone 22-13) and the copy number of the synthetic chromosome are known
15 (Table 2), the amount, in kilobases, of synthetic alpha satellite DNA (Figure 7B) can be estimated.

 Although it is difficult to estimate the overall size of these synthetic chromosomes, in some cases, the synthetic chromosome is barely detectable using a fluorescence microscope at 1000x magnification.

20 *Synthetic chromosome structure and copy number*

 Upon initial analysis, each clone of synthetic chromosome containing cells possessed very few synthetic chromosomes, and in most cases, only one per cell. This shows that the copy number of the synthetic chromosomes is regulated like that of the endogenous chromosomes.

25 In addition to copy number, the synthetic chromosomes share two other features with the endogenous chromosomes. First, they contain telomeric

sequences (data not shown). This suggests that these synthetic chromosomes are linear. Second, in metaphase chromosomes, the individual chromatids are clearly visible on each synthetic chromosome (Figures 6-7). This shows that the overall structure of the synthetic chromosome is similar to that of the endogenous chromosomes. Furthermore, since chromatids are normally held together at the centromere, this result also shows that the synthetic chromosomes are capable of carrying out at least one centromeric function, the attachment of sister chromatids.

CENP-E associates with synthetic chromosomes during metaphase

The presence of synthetic chromosomes (in most cases at single copy) in dividing cells shows the creation of a functional centromere. In order to further investigate this, several of the synthetic chromosomes were tested to determine whether CENP-E was present at the centromere during metaphase. It has been shown previously that CENP-B is present at both functional and nonfunctional centromeres (Earnshaw, W.C. *et al.*, *Chromosoma* 98:1-12 (1989)), and therefore, it can not be used as a marker for centromere activity. For this reason, CREST antisera (used in previous experiments: Haaf *et al.*, Larin *et al.*, and Praznovsky *et al.* (cited above)), which generally recognizes CENP-B very strongly, is not a good reagent for assessing centromere activity. On the other hand, CENP-E has been shown to be present only at functional centromeres (Sullivan & Schwartz, *Hum. Mol. Genet.* 4:2189-2197 (1995)), and therefore, monospecific antibodies to this protein can be used to assess centromere activity.

Consistent with the presence of a functional centromere, it was found that CENP-E was present on the synthetic chromosome in clones 22-11 and 23-1, the only clones tested to date (Figure 9). Furthermore, the amount of CENP-E on the synthetic chromosome is similar to that present at the centromere of each of the endogenous chromosomes. This is interesting because CENP-E is not thought to bind to centromeric DNA directly, and therefore, its level does not depend on

the amount of alpha satellite present. Instead, it depends solely on whether a functional kinetochore has formed. Thus, the presence of CENP-E on the synthetic chromosome during metaphase strongly suggests that they contain a functional centromere capable of directing formation of a centromere/kinetochore complex.

Synthetic chromosomes are mitotically stable in the absence of selection

To confirm that the synthetic chromosomes contain a functional centromere and are capable of correctly segregating in dividing cells, the synthetic chromosome containing cells were grown for a defined period of time in the absence of selection. The cells were then analyzed by FISH to determine the percentage of cells that contained the synthetic chromosome. After 46 days (approximately 60 cell generations) in the absence of selection, the synthetic chromosomes were still present in the majority of cells (Table 2). In several clones, the synthetic chromosome was still present in 100% of the cells. This indicates that the synthetic chromosomes are mitotically stable, and therefore, validates the idea that these vectors can be used to transfect dividing cells to correct genetic defects *in vivo*.

In addition to determining the segregation efficiency of each synthetic chromosome, this experiment also allowed us to assess the structural stability of the synthetic chromosomes over time. After scanning 50 chromosome spreads for each clone, no cases in which the synthetic chromosome integrated into an endogenous chromosome were observed. Furthermore, no other gross rearrangements involving the synthetic chromosomes were observed. This result, in conjunction with their high degree of mitotic stability, demonstrates that these synthetic chromosomes behave as separate genetic units with many of the same characteristics as endogenous human chromosomes.

Gene expression from the synthetic chromosomes

The synthetic chromosomes described here provide an alternative vector for somatic gene therapy. It is, therefore, important to determine whether heterologous genes can be efficiently expressed from these chromosome vectors.

5 As described in the experimental procedures, the synthetic chromosomes were created by co-transfecting pVJ104-Y α 16 or pVJ104-17 α 32 with telomeric DNA and human genomic DNA into HT1080 cells. In each transfection, the β -geo expression unit was linked to at least 100 kb of alpha satellite DNA. Following transfection, the location of alpha satellite in the cell is the same as the
10 location of the β -geo gene. Thus, in the synthetic chromosome clones, with the exception of clone 17-15, the β -geo gene is located exclusively on the synthetic chromosome.

To determine the levels of β -geo expression in each of the synthetic chromosome containing clones, and therefore the extent of gene expression from
15 the synthetic chromosome, the cells were assayed using the X-gal plate staining method described in the experimental procedures. Although this technique is relatively insensitive (i.e. β -geo expression must be high in order to be detected), it provided a rough approximation of expression levels and the percentage of cells expressing this marker gene. After 70 days in culture without G418 selection
20 (approximately 80 cell divisions), at least 50% of the cells in clones 22-11 expressed β -geo at levels detectable in this assay (Figure 10). In clone 22-6, approximately 25% of the cells had detectable β -geo activity after 70 days in the absence of selection (data not shown). Expression in the other clones could not be evaluated due to the insensitivity of this assay and due to the lower expression
25 of β -geo in these cells.

Discussion

The results show that naked DNA can be transfected into mammalian cells and, without integrating into an endogenous chromosome, form a functional synthetic chromosome. These synthetic chromosomes have many of the characteristics of normal mammalian chromosomes. First, they are present in the cell at low copy number, usually one per cell. Second, the synthetic chromosomes appear to be linear and contain telomeric sequences. Third, during mitosis, CENP-E is bound to the synthetic chromosome indicating the formation of a functional kinetochore. Fourth, the synthetic chromosomes are mitotically stable in the absence of selection. Fifth, the synthetic chromosomes are capable of harboring transcriptionally active genes. Finally, the synthetic chromosomes are structurally stable over time, with an undetectable integration frequency. Unlike normal human chromosomes, the synthetic chromosomes are small and easily manipulated allowing different genes to be expressed in a variety of chromosomal contexts.

The results show *in vitro* methods for producing alpha satellite arrays up to 736 kb in length. In addition to providing an essential component to the synthetic chromosomes described here, these results demonstrate that alpha satellite can be produced in clinically useful quantities. Previously, there has been no method available allowing structurally intact alpha satellite DNA greater than 200 kb to be purified in the quantities necessary for the transfection of mammalian cells.

As a control, the inventors recreated the previous failed experiments of Haaf *et. al.*, creating a chromosome with the concomitant integration of α satellite DNA into an endogenous chromosome. The transfection in which this occurred lacked additional genomic DNA sequences. Without genomic sequences, it is very likely that the chromosome formed as a result of a breakage event from one of the endogenous chromosomes that contain integrated alpha satellite DNA. In addition to being an inefficient and infrequent event, this approach is not useful

for gene therapy procedures due to the risk of inducing genomic rearrangement and malignant transformation in the host cell as a result of the chromosome breakage mechanism.

5 In summary, the inventors have demonstrated that mitotically stable synthetic chromosomes can be created by transfecting large alpha satellite arrays, telomeric DNA, and genomic DNA together into a human cell. Although each of these components appears to be necessary for efficient synthetic chromosome formation, it is possible that genomic rearrangements following integration of alpha satellite DNA can lead to chromosome formation in the absence of genomic
10 (non-alpha satellite, non-telomeric) sequences. On the other hand, alpha satellite DNA appears to be absolutely required to produce these synthetic chromosomes. Here, by creating synthetic chromosomes using alpha satellite derived from the Y chromosome and from chromosome 17, the inventors have demonstrated that the source of alpha satellite DNA is not important. In other words, alpha satellite
15 DNA from any chromosome can be used to create synthetic chromosomes. Furthermore, given that human chromosomes are stable in a variety of hybrids, including mouse, hamster, and primate, alpha satellite-like sequences from these other species can also be used to create synthetic chromosomes such as those described here.

Table 1

Clone	Y α	17 α	hg	tel	VK75	Characteristics
C10-7	XX			X		Integrand (mid-Q of a large chromosome)
C10-10	XX			X		Integrand (mid arm-medium size chromosome)
C11-7		X		X		Integrated (forms a 17p+)
C11-19		X		X		no signal
C12-2		XX		X		no signal
C12-3		XX		X		no signal
C12-5		XX		X		Integrated (mid arm)
C12-6		XX		X		Integrated (non-telomeric)
C12-14		XX		X		no signal
C12-16		XX		X		no signal
C13-1				X	X	Integrated
C14-1	X			X	X	Telomere directed truncation
C15-2	X			X		Chr. 6 truncation; de novo telomere
C15-3	X			X		no signal
C15-4	X			X		Integrated (p arm of a 16 like chrom)
C15-5	X			X		no signal
C15-10	X			X		Integrated into a telocentric chromosome
C15-12	X			X		Integrated into chrom 17 below centromere
C15-13	X			X		no signal
C15-21	X			X		no signal
C16-6	XX			X		Telomeric/telocentric p-constriction
C16-7	XX			X		no signal
C17-2		X		X		no signal
C17-8		X		X		no signal

Clone	Y α	17 α	hg	tel	VK75	Characteristics
C17-11		X		X		truncation of C group chromosome
C17-15		X		X		microchromosome, truncation of C group chromosome
C17-19		X		X		no signal
C19-1	X		X	X		no signal
C19-2	X		X	X		Ambiguous (telomere directed truncation?)
C21-1			X	X	X	no signal
C22-2	(XX)		X	X		Chr. truncation (<i>de novo</i> telomere @ 19p)
C22-3	(XX)		X	X		no signal
C22-4	(XX)		X	X		Telomeric/Possible dicentric
C22-5	(XX)		X	X		Ambiguous; very small micro?
C22-6	(XX)		X	X		Double micro (multiple micro)
C22-7	(XX)		X	X		Large micro
C22-8	(XX)		X	X		Ambiguous (possible micro)
C22-9	(XX)		X	X		Telomeric/large array
C22-11	(XX)		X	X		Small micro
C22-13	(XX)		X	X		Large micro
C23-1		(XX)	X	X		Small micro

Table 1. Results from the transfection of various combinations of alpha satellite DNA, telomeric DNA, and human genomic DNA into human cells. Y α and 17 α are abbreviations for alpha satellite from the Y chromosome and chromosome 17, respectively. hg is an abbreviation for human genomic DNA that was digested with *NotI*. Tel is an abbreviation for telomeric DNA. VK75 is an abbreviation for a 75 kb fragment from the X chromosome. X indicates that a sequence was included in the transfection. XX indicates that additional purified alpha satellite DNA was included in the transfection, as described in the Experimental Procedures. (XX) indicates that additional alpha satellite DNA was preligated to the b-geo/alpha satellite construct prior to transfection, as described in the Experimental Procedures.

Table 2

CLONE	t = 0 days	t = 10 days	t = 20 days	t = 46 days
22-7 (Y)	10 cells: 2 Ys	10 cells: 2 Ys (7 cells)	10 cells: 2 Ys (9 cells)	10 cells: 2 Ys (8 cells)
Microchromosome	1 microchromosome:	1 microchromosome:	1 microchromosome:	1 microchromosome:
	100%	100%	100%	100%
22-11 (Y)	20 cells: 2 Ys (17 cells)	10 cells: 2 Ys (6 cells)	10 cells: 2 Ys (8 cells)	10 cells: 2 Ys (5 cells)
Microchromosome	t(Y;14) (19 cells)	t(Y;14) (9 cells)	t(Y;14) (10 cells)	1 Y (4 cells)
	0 microchromosomes (7 cells)	13 p+ (8-9 cells)	13 p+ (8+ cells)	0 Y (1 cell)
	1 microchromosome (12 cells)	1 microchromosome (2 cells)	0 microchromosomes* (2 cells)	0 microchromosomes (5 cells)
	2 microchromosomes (1 cell)	2 microchromosomes (8 cells)	1 microchromosome (2 cells)	1 microchromosome (2-3 cells)
	65%	100%	2 microchromosomes (5 cells)	2 microchromosomes (2 cells)
			3 microchromosomes (1 cell)	40-50%
			?80+%	
22-13 (Y)	17 cells: 1 Y	10 cells: 1 Y	10 cells: 1 Y	10 cells: 1 Y
Microchromosome	1 microchromosome:	1 microchromosome:	1 microchromosome:	1 microchromosome:
	100%	100%	100%	100%

CLONE	t = 0 days	t = 10 days	t = 20 days	t = 46 days
23-1 (17)	11 cells: 4-17s	10 cells: 4-17s	10 cells: 4-17s	10 cells: 4-17s
Microchromosome	1 microchromosome:	1 microchromosome:	1 microchromosome:	1 microchromosome:
	100%	100%	100%	100%
17-15 (17)	17 cells: 4-17s (15 cells)	N.D.	10 cells: 4-17s	N.D.
Microchromosome	1 Cqter (10 cells)		1 Cqter (6 cells)	
	1 microchromosome:		1 microchromosome:	
	100%		100%	
22-6 (Y)	9 cells: 2 Ys	10 cells: 2 Ys	10 cells: 2 Ys (7 cells)	N.D.
Integrated signals	1 metacent, huge sig. (2 cells)	1 metacent, huge sig. (2 cells)	1 metacent, huge sig. (5 cells)	
	1 D-like w/ signal (2 cells)	1 D-like w/ signal (1 cell)	1 E-like (?) w/ signal (1 cell)	
* both spreads were broken				

Table 2. The synthetic chromosome is mitotically stable in the absence of selection. Each clone was grown in the absence of selection for the indicated number of days. Following this culture period, the cells were harvested for FISH and hybridized to the appropriate alpha satellite probe as described in the Experimental Procedures herein. For each time point, the number of cells analyzed, the number of Y chromosomes (or the number of number 17 chromosomes in the cases of clone 23-1 and 17-15), and the number of synthetic chromosomes are indicated. In addition, the percentage of cells containing a synthetic chromosome is shown in bold for each time point. The time points 0, 10, and 20 days correspond to 0, 12, and 24 cell divisions, respectively.

Although the foregoing refers to particular preferred embodiments, it will be understood that the present invention is not so limited. It will occur to those ordinarily skilled in the art that various modifications may be made to the disclosed embodiments and that such modifications are intended to be within the scope of the present invention.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANTS:

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- (B) HARRINGTON, John J.
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(ii) TITLE OF INVENTION: Synthetic Mammalian Chromosome
And Methods For Construction

(iii) NUMBER OF SEQUENCES: 5

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- (D) STATE: D.C.
- (E) COUNTRY: U.S.A.
- (F) ZIP: 20005-3934

(v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: Patent In Release #1.0, Version #1.30 (EPO)

(vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER: (To Be Advised)
- (B) FILING DATE: 07-JUN-1996

(vii) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBERS: US 08/487,989 AND US 08/643,554
- (B) FILING DATES: 07-JUN-1995 AND 06-MAY-1996

(viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: MICHELE A. CIMBALA
- (B) REGISTRATION NUMBER: 33,851
- (C) REFERENCE/DOCKET NUMBER: 1522.0001PC02

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

-72-

ATTCGTTGGA AACGGGA

17

(2) INFORMATION FOR SEQ ID NO: 2:

(I) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GGGCGGGAGA TCTCAGAAAA TTCTTTGGGA TGATTGAGTT G
41

(2) INFORMATION FOR SEQ ID NO: 3:

(I) SEQUENCE CHARACTERISTICS:

- 15 (A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GGGCGGGATC CCTTCTGTCT TCTTTTATA GGAAGTTATT T
41

(2) INFORMATION FOR SEQ ID NO: 4:

(I) SEQUENCE CHARACTERISTICS:

- 25 (A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

-73-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GGGTTAGGGT TAGGGTAGG GTTAGGGTTA GGGTTAGGG
39

(2) INFORMATION FOR SEQ ID NO: 5:

5

- (I) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

10

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CCCTAACCCT AACCTAACC CTAACCCTAA CCCTAACCC
39

What Is Claimed Is:

1. An artificial mammalian chromosome comprising essentially centromeric, telomeric, and genomic DNA.

2. An artificial mammalian chromosome comprising essentially centromeric DNA, telomeric DNA, and genomic DNA, wherein said genomic DNA is a sub-genomic DNA fragment selected from the group consisting of restriction enzyme digestion fragments and mechanically-sheared fragments.

3. An artificial mammalian chromosome comprising essentially centromeric DNA, telomeric DNA, and genomic DNA, wherein said genomic DNA is a sub-genomic DNA fragment selected from the group consisting of restriction enzyme digestion fragments and mechanically-sheared fragments, said centromeric DNA comprises a DNA sequence that associates with CENP-E during mitosis, and said telomeric DNA comprises tandem repeats of the sequence TTAGGG.

4. An artificial mammalian chromosome produced by the process of transfecting a mammalian cell with purified DNA, said DNA comprising essentially telomeric DNA, centromeric DNA, and genomic DNA, wherein said genomic DNA is a sub-genomic DNA fragment selected from the group consisting of restriction enzyme digestion fragments and mechanically-sheared fragments.

5. An artificial mammalian chromosome produced by the process of transfecting a mammalian cell with purified DNA, said DNA comprising essentially telomeric DNA, centromeric DNA, and genomic DNA, wherein said genomic DNA is a sub-genomic DNA fragment selected from the group

consisting of restriction enzyme digestion fragments and mechanically-sheared fragments, said centromeric DNA comprises a DNA sequence that associates with CENP-E during mitosis, and said telomeric DNA comprises tandem repeats of the sequence TTAGGG.

5 6. An artificial mammalian chromosome produced by the process of transfecting a mammalian cell with purified naked DNA, said DNA comprising essentially telomeric DNA, centromeric DNA, and genomic DNA, wherein said genomic DNA is a sub-genomic DNA fragment selected from the group consisting of restriction enzyme digestion fragments and mechanically-sheared
10 fragments.

 7. An artificial mammalian chromosome produced by the process of transfecting a mammalian cell with purified naked DNA, said DNA comprising essentially telomeric DNA, centromeric DNA, and genomic DNA, wherein said genomic DNA is a sub-genomic DNA fragment selected from the group
15 consisting of restriction enzyme digestion fragments and mechanically-sheared fragments, said centromeric DNA comprises a DNA sequence that associates with CENP-E during mitosis, and said telomeric DNA comprises tandem repeats of the sequence TTAGGG.

 8. An artificial mammalian chromosome produced by the process of transfecting a mammalian cell with purified condensed DNA, said DNA comprising essentially telomeric DNA, centromeric DNA, and genomic DNA,
20 wherein said genomic DNA is a sub-genomic DNA fragment selected from the group consisting of restriction enzyme digestion fragments and mechanically-sheared fragments.

5 9. An artificial mammalian chromosome produced by the process of transfecting a mammalian cell with purified condensed DNA, said DNA comprising essentially telomeric DNA, centromeric DNA, and genomic DNA, wherein said genomic DNA is a sub-genomic DNA fragment selected from the group consisting of restriction enzyme digestion fragments and mechanically-sheared fragments, said centromeric DNA comprises a DNA sequence associates with binds CENP-E during mitosis, and said telomeric DNA comprises tandem repeats of the sequence TTAGGG.

10 10. An artificial mammalian chromosome produced by the process of transfecting purified coated DNA into a mammalian cell, said DNA comprising essentially a centromere, a telomere, and genomic DNA, wherein said genomic DNA is a sub-genomic DNA fragment selected from the group consisting of restriction enzyme digestion fragments and mechanically-sheared fragments.

15 11. An artificial mammalian chromosome produced by the process of transfecting purified coated DNA into a mammalian cell, said DNA comprising essentially a centromere, a telomere, and genomic DNA, wherein said genomic DNA is a sub-genomic DNA fragment selected from the group consisting of restriction enzyme digestion fragments and mechanically-sheared fragments, said centromere comprises a DNA sequence that associates with CENP-E during mitosis, and said telomere comprises tandem repeats of the sequence TTAGGG.

20 12. The artificial mammalian chromosome of any of claims 4-11, wherein said centromeric DNA, said telomeric DNA and said genomic DNA are not ligated to each other.

13. The artificial mammalian chromosome of any of claims 4-11, wherein one or more of said centromeric DNA, said telomeric DNA and said genomic DNA are ligated to one another.

14. A composition comprising the artificial mammalian chromosome of any of claims 1-11.

15. The artificial mammalian chromosome of any of claims 1-11, wherein said centromeric DNA comprises alpha-satellite DNA.

16. A mammalian cell comprising the artificial mammalian chromosome of any of claims 1-11.

17. The artificial mammalian chromosome of any of claims 1-11, wherein said chromosome further comprises a heterologous DNA that is expressed from said chromosome, or causes expression of a gene product, when said chromosome is introduced into a mammalian cell.

18. Purified DNA comprising essentially telomeric DNA, centromeric DNA, and genomic DNA, wherein said genomic DNA is a sub-genomic DNA fragment selected from the group consisting of restriction enzyme digestion fragments and mechanically-sheared fragments.

19. Purified DNA comprising essentially telomeric DNA, centromeric DNA, and genomic DNA, wherein said genomic DNA is a sub-genomic DNA fragment selected from the group consisting of restriction enzyme digestion fragments and mechanically-sheared fragments, said centromeric DNA comprises a DNA sequence that associates with CENP-E during mitosis, and said telomeric DNA comprises tandem repeats of the sequence TTAGGG.

20. Purified naked DNA comprising essentially telomeric DNA, centromeric DNA, and genomic DNA, wherein said genomic DNA is a sub-genomic DNA fragment selected from the group consisting of restriction enzyme digestion fragments and mechanically-sheared fragments.

5 21. Purified naked DNA comprising essentially telomeric DNA, centromeric DNA, and genomic DNA, wherein said genomic DNA is a sub-genomic DNA fragment selected from the group consisting of restriction enzyme digestion fragments and mechanically-sheared fragments, said centromeric DNA comprises a DNA sequence that associates with CENP-E during mitosis, and said
10 telomeric DNA comprises tandem repeats of the sequence TTAGGG.

22. Purified condensed DNA comprising essentially telomeric DNA, centromeric DNA, and genomic DNA, wherein said genomic DNA is a sub-genomic DNA fragment selected from the group consisting of restriction enzyme digestion fragments and mechanically-sheared fragments, wherein said DNA is
15 coated with a DNA-condensing agent.

23. Purified condensed DNA comprising essentially telomeric DNA, centromeric DNA, and genomic DNA, wherein said genomic DNA is a sub-genomic DNA fragment selected from the group consisting of restriction enzyme digestion fragments and mechanically-sheared fragments, said centromeric DNA
20 comprises a DNA sequence that associates with CENP-E during mitosis, and said telomeric DNA comprises tandem repeats of the sequence TTAGGG, wherein said DNA is combined with a DNA-condensing agent.

24. Purified coated DNA comprising essentially telomeric DNA, centromeric DNA, and genomic DNA, wherein said genomic DNA is a sub-genomic DNA fragment selected from the group consisting of restriction enzyme
25

digestion fragments and mechanically-sheared fragments, wherein said DNA is coated with one or more DNA-binding proteins.

5 25. Purified coated DNA comprising essentially telomeric DNA, centromeric DNA, and genomic DNA, wherein said genomic DNA is a sub-genomic DNA fragment selected from the group consisting of restriction enzyme digestion fragments and mechanically-sheared fragments, said centromeric DNA comprises a DNA sequence that associates with CENP-E during mitosis, and said telomeric DNA comprises tandem repeats of the sequence TTAGGG, wherein said DNA is coated with one or more DNA-binding proteins.

10 26. Purified DNA made by the process of combining, *in vitro*, telomeric DNA, centromeric DNA, and genomic DNA, wherein said genomic DNA is a sub-genomic DNA fragment selected from the group consisting of restriction enzyme digestion fragments and mechanically-sheared fragments.

15 27. Purified DNA made by the process of combining, *in vitro*, telomeric DNA, centromeric DNA, and genomic DNA, wherein said genomic DNA is a sub-genomic DNA fragment selected from the group consisting of restriction enzyme digestion fragments and mechanically-sheared fragments, said centromeric DNA associates with CENP-E during mitosis, and said telomeric DNA comprises tandem repeats of the sequence TTAGGG.

20 28. Purified naked DNA made by the process of combining, *in vitro*, telomeric DNA, centromeric DNA, and genomic DNA, wherein said genomic DNA is a sub-genomic DNA fragment selected from the group consisting of restriction enzyme digestion fragments and mechanically-sheared fragments.

29. Purified naked DNA made by the process of combining, *in vitro*, telomeric DNA, centromeric DNA, and genomic DNA, wherein said genomic DNA is a sub-genomic DNA fragment selected from the group consisting of restriction enzyme digestion fragments and mechanically-sheared fragments, said
5 centromeric DNA associates with CENP-E during mitosis, and said telomeric DNA comprises tandem repeats of the sequence TTAGGG.

30. Purified condensed DNA made by the process of combining, *in vitro*, telomeric DNA, centromeric DNA, and genomic DNA, wherein said genomic DNA is a sub-genomic DNA fragment selected from the group
10 consisting of restriction enzyme digestion fragments and mechanically-sheared fragments, wherein said DNA is combined with a DNA-condensing agent.

31. Purified condensed DNA made by the process of combining, *in vitro*, telomeric DNA, centromeric DNA, and genomic DNA, wherein said genomic DNA is a sub-genomic DNA fragment selected from the group
15 consisting of restriction enzyme digestion fragments and mechanically-sheared fragments, said centromeric DNA associates with CENP-E during mitosis, and said telomeric DNA comprises tandem repeats of the sequence TTAGGG, wherein said DNA is combined with a DNA-condensing agent.

32. Purified coated DNA made by the process of combining, *in vitro*, telomeric DNA, centromeric DNA, and genomic DNA, wherein said genomic
20 DNA is a sub-genomic DNA fragment selected from the group consisting of restriction enzyme digestion fragments and mechanically-sheared fragments, wherein said DNA is coated with one or more DNA-binding proteins.

33. Purified coated DNA made by the process of combining, *in vitro*, telomeric DNA, centromeric DNA, and genomic DNA, wherein said genomic
25

DNA is a sub-genomic DNA fragment selected from the group consisting of restriction enzyme digestion fragments and mechanically-sheared fragments, said centromeric DNA associates with CENP-E during mitosis, and said telomeric DNA comprises tandem repeats of the sequence TTAGGG, wherein said DNA is coated with one or more DNA-binding proteins.

34. A composition comprising the DNA of any of claims 18-33.

35. A mammalian cell comprising the purified DNA of any of claims 18-33.

36. The purified DNA of any of claims 18-33, wherein said centromeric DNA, said telomeric DNA and said genomic DNA are not ligated to each other.

37. The purified DNA of any of claims 18-33, wherein one or more of said centromeric DNA, said telomeric DNA and said genomic DNA are ligated to each other.

38. The purified DNA of any of claims 18-33, wherein said centromeric DNA comprises alpha-satellite DNA.

39. The purified DNA of any of claims 18-33, wherein said DNA further comprises heterologous DNA that is expressed from said chromosome, or causes expression of a gene product, when said DNA is introduced into a mammalian cell.

40. A vector comprising the DNA of any of claims 18-33.

41. A cell comprising the vector of claim 40.

42. A composition comprising the vector of claim 40.

43. A method of cloning repeating tandem arrays of DNA, said method comprising

- 5 (a) preparing a first DNA unit such that the opposing ends of said DNA unit contain complementary, but non-isoschizomeric restriction sites;
- (b) ligating said DNA unit into a vector;
- (c) linearizing said vector at one of said restriction sites;
- 10 (d) ligating a second DNA unit, prepared as in step (a), in tandem with said first unit, so as to form a directional, repeating array;
- (e) transforming said array into a bacterial host cell;
- (f) selecting stable clones containing said array; and
- 15 (g) repeating steps (c)-(f) until a desired array size is reached.

44. The method of claim 43, wherein said DNA is alpha satellite DNA.

45. The method of claim 44, wherein said array of said alpha satellite DNA is greater than 100 kb in length.

20 46. The method of claim 45, wherein said array of said alpha satellite DNA is greater than 140 kb in length.

47. The method of claim 44, wherein said alpha satellite DNA is human alpha satellite DNA.

48. A vector comprising a sequence consisting of a directional, repeating, DNA array, said array comprising repeating DNA units, wherein the opposing ends of each DNA unit contain complementary, but non-isoschizomeric restriction sites.

5 49. The vector of claim 48, wherein said DNA is alpha satellite DNA.

50. The vector of claim 49, wherein said array of said alpha satellite DNA is greater than 100 kb in length.

51. The vector of claim 50, wherein said array of said alpha satellite DNA is greater than 140 kb in length.

10 52. The vector of claim 47, wherein said alpha satellite DNA is human alpha satellite DNA.

53. A host cell stably transformed with the vector of any one of claims 47-52.

15 54. The host cell of claim 53, wherein said host cell is a prokaryotic cell.

55. The host cell of claim 54, wherein said prokaryotic cell is *E. coli*.

56. A method of making an artificial mammalian chromosome, said method comprising introducing the purified DNA of any of claims 18-33 into a mammalian cell.

57. A method of making an artificial mammalian chromosome, said method comprising introducing the composition of claim 34 into a mammalian cell.

5 58. A method of making a purified DNA composition, said method comprising combining, *in vitro*, purified telomeric DNA, centromeric DNA, and genomic DNA, wherein said genomic DNA is a sub-genomic DNA fragment selected from the group consisting of restriction enzyme digestion fragments and mechanically-sheared fragments.

10 59. A method of making a purified DNA composition, said method comprising combining, *in vitro*, purified telomeric DNA, centromeric DNA, and genomic DNA, wherein said genomic DNA is a sub-genomic DNA fragment selected from the group consisting of restriction enzyme digestion fragments and mechanically-sheared fragments, said centromeric DNA comprises a DNA sequence that associates with CENP-E during mitosis, and said telomeric DNA
15 comprises tandem repeats of the sequence TTAGGG.

60. A method of making a purified naked DNA composition, said method comprising combining, *in vitro*, purified telomeric DNA, centromeric DNA, and genomic DNA, wherein said genomic DNA is a sub-genomic DNA fragment selected from the group consisting of restriction enzyme digestion
20 fragments and mechanically-sheared fragments.

61. A method of making a purified naked DNA composition, said method comprising combining, *in vitro*, purified telomeric DNA, centromeric DNA, and genomic DNA, wherein said genomic DNA is a sub-genomic DNA fragment selected from the group consisting of restriction enzyme digestion
25 fragments and mechanically-sheared fragments, said centromeric DNA comprises

a DNA sequence that associates with CENP-E during mitosis, and said telomeric DNA comprises tandem repeats of the sequence TTAGGG.

5 62. A method of making a purified condensed DNA composition, said method comprising combining, *in vitro*, a DNA-condensing agent and purified telomeric DNA, centromeric DNA, and genomic DNA, wherein said genomic DNA is a sub-genomic DNA fragment selected from the group consisting of restriction enzyme digestion fragments and mechanically-sheared fragments.

10 63. A method of making a purified condensed DNA composition, said method comprising combining, *in vitro*, a DNA-condensing agent and purified telomeric DNA, centromeric DNA, and genomic DNA, wherein said genomic DNA is a sub-genomic DNA fragment selected from the group consisting of restriction enzyme digestion fragments and mechanically-sheared fragments, said centromeric DNA comprises a DNA sequence that associates with CENP-E during mitosis, and said telomeric DNA comprises tandem repeats of the
15 sequence TTAGGG.

20 64. A method of making a purified coated DNA composition, said method comprising combining, *in vitro*, one or more DNA-binding proteins and purified telomeric DNA, centromeric DNA, and genomic DNA, wherein said genomic DNA is a sub-genomic DNA fragment selected from the group consisting of restriction enzyme digestion fragments and mechanically-sheared fragments.

25 65. A method of making a purified coated DNA composition, said method comprising combining, *in vitro*, one or more DNA-binding proteins and purified telomeric DNA, centromeric DNA, and genomic DNA, wherein said genomic DNA is a sub-genomic DNA fragment selected from the group

consisting of restriction enzyme digestion fragments and mechanically-sheared fragments, said centromeric DNA comprises a DNA sequence that associates with CENP-E during mitosis, and said telomeric DNA comprises tandem repeats of the sequence TTAGGG.

5 66. The method of any of claims 56-65, wherein said centromeric DNA, said telomeric DNA and said genomic DNA are not ligated to each other.

67. The method of any of claims 56-65, wherein one or more of said centromeric DNA, said telomeric DNA and said genomic DNA are ligated to each other.

10 68. A method of expressing a gene in a mammalian cell, said method comprising propagating a mammalian cell containing the artificial chromosome of any of claims 1-11, wherein said chromosome contains said gene or contains a DNA sequence that allows expression of said gene.

15 69. A method of expressing a heterologous gene in a mammalian cell, said method comprising propagating a mammalian cell containing the DNA of any of claims 18-33, wherein said DNA contains said gene or contains a DNA sequence that allows expression of said gene.

70. The method of claim 68, wherein said gene expression provides a therapeutic benefit to a mammal comprising said cell.

20 71. The method of claim 69, wherein said gene expression provides a therapeutic benefit to a mammal comprising said cell.

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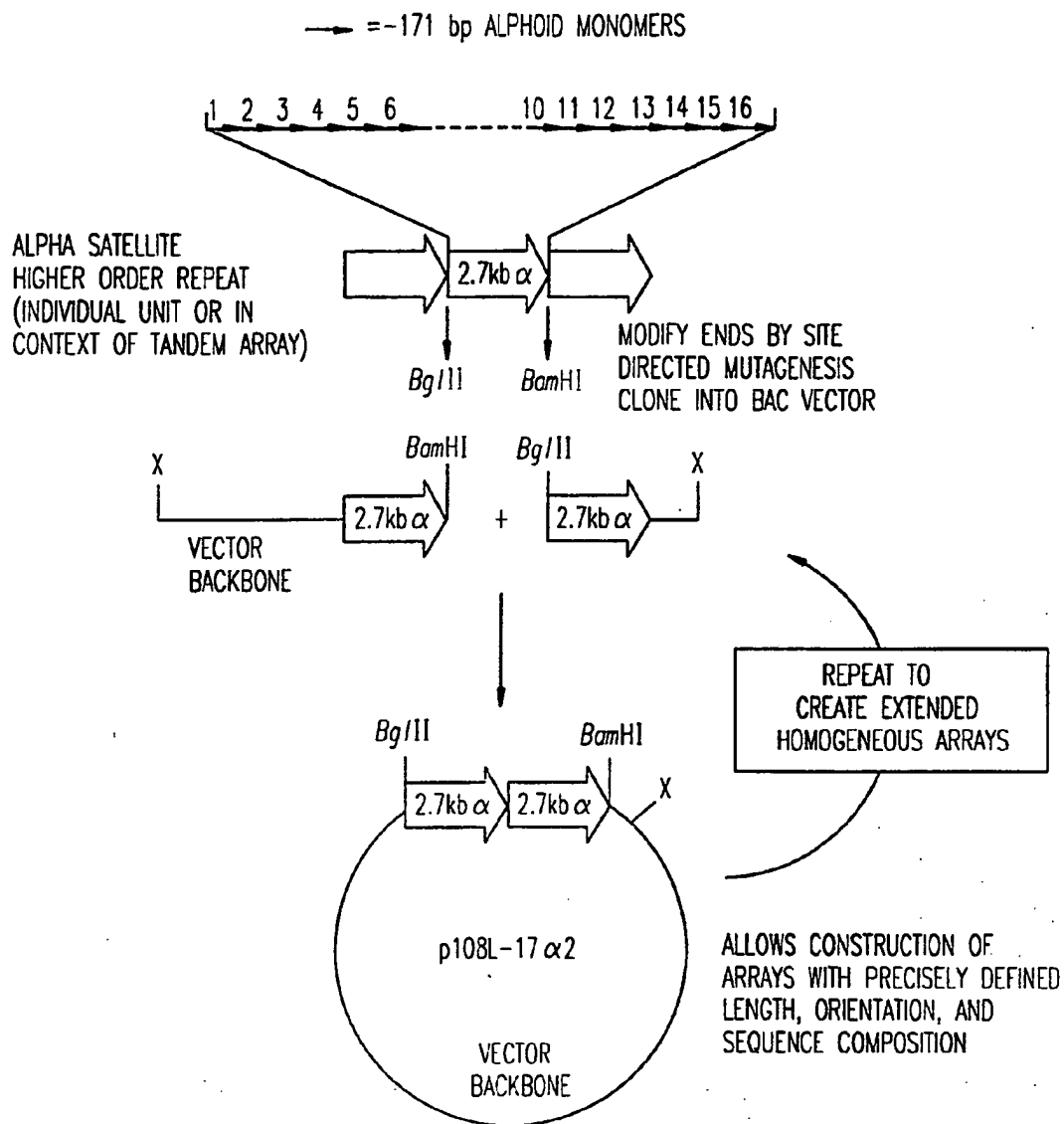


FIG.1

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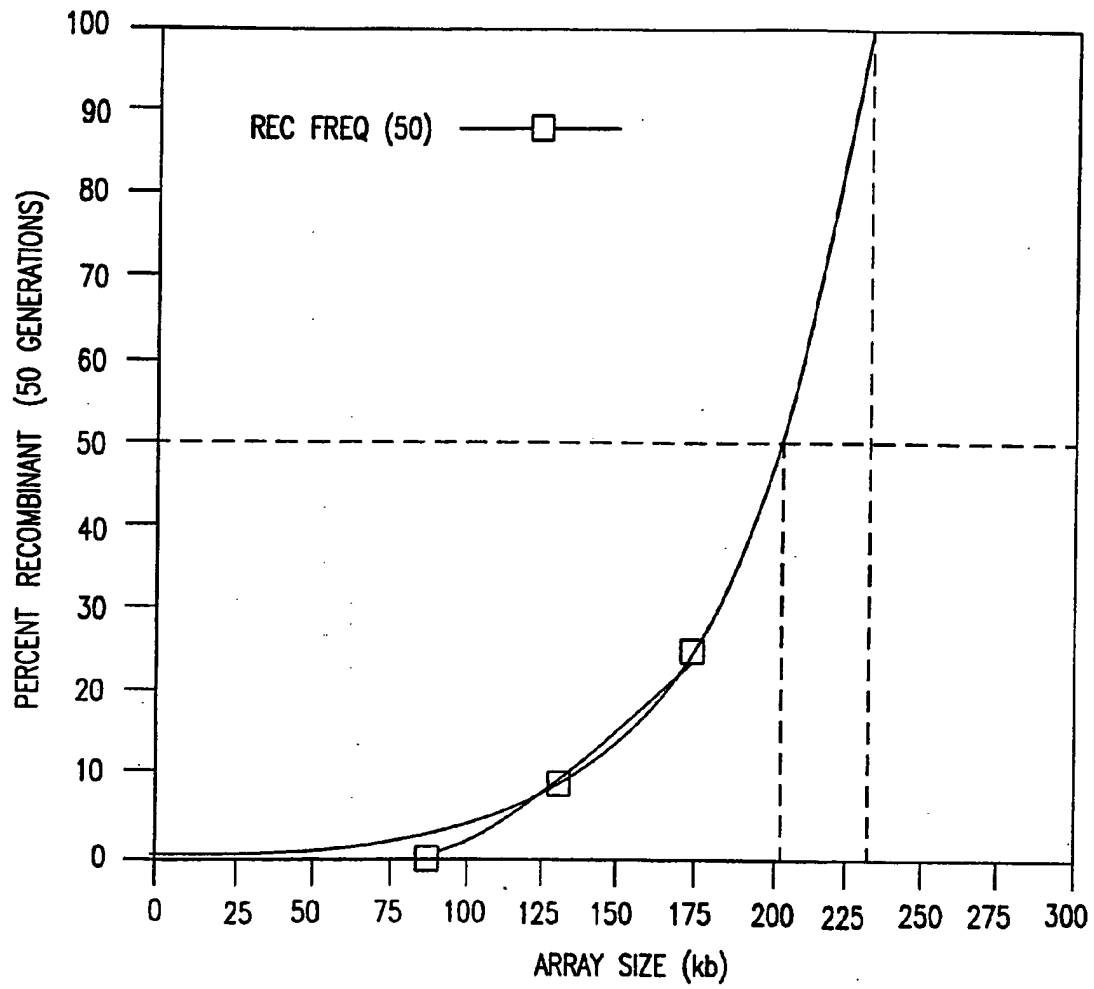


FIG.2

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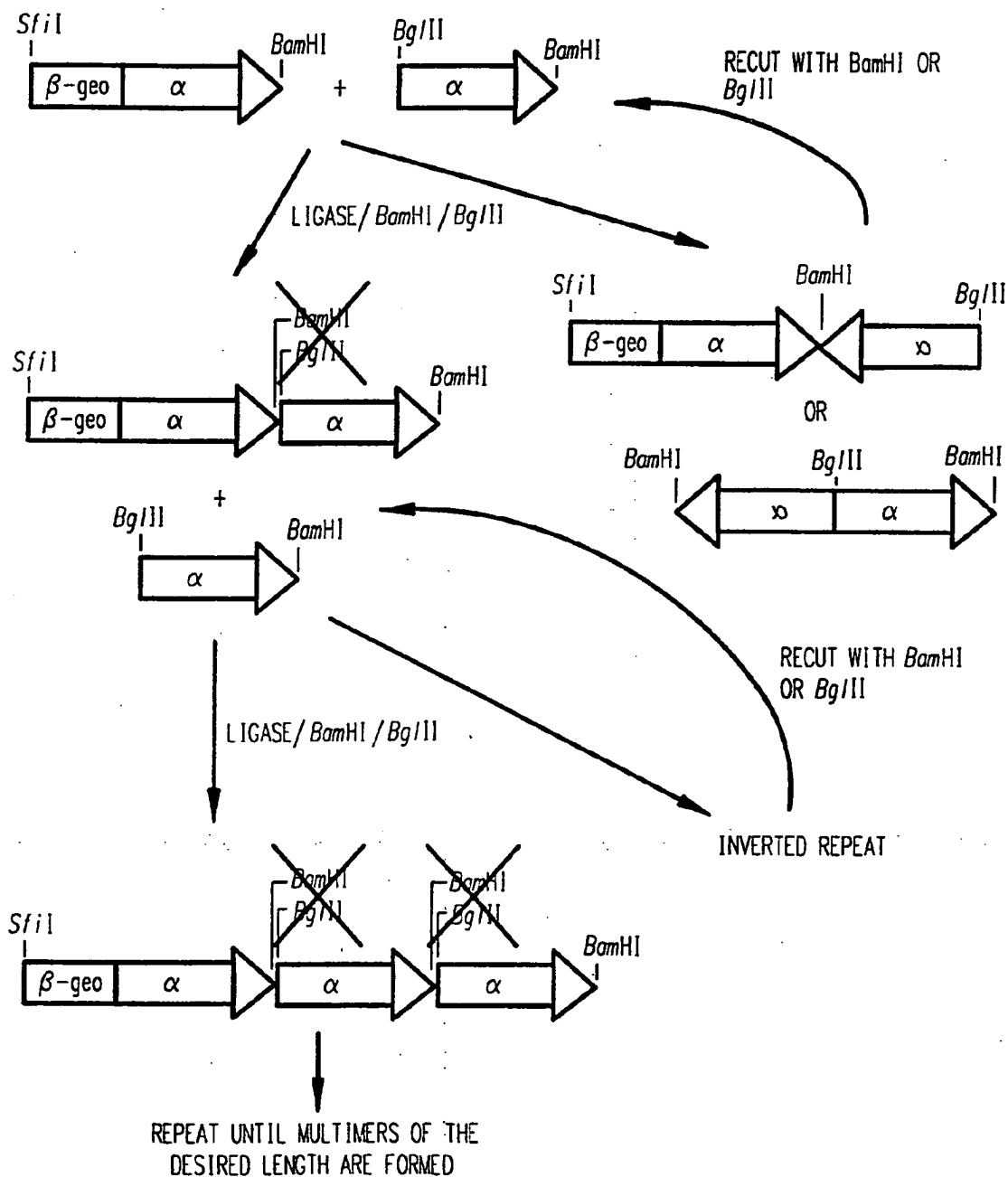


FIG.3A
SUBSTITUTE SHEET (RULE 26)

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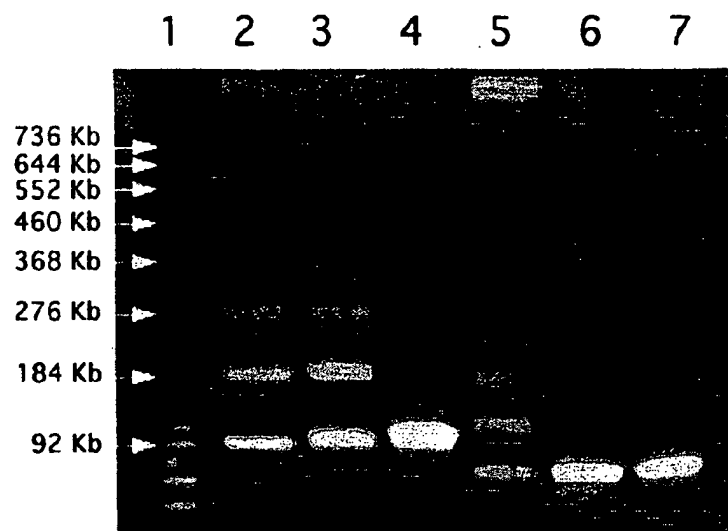


FIG.3B

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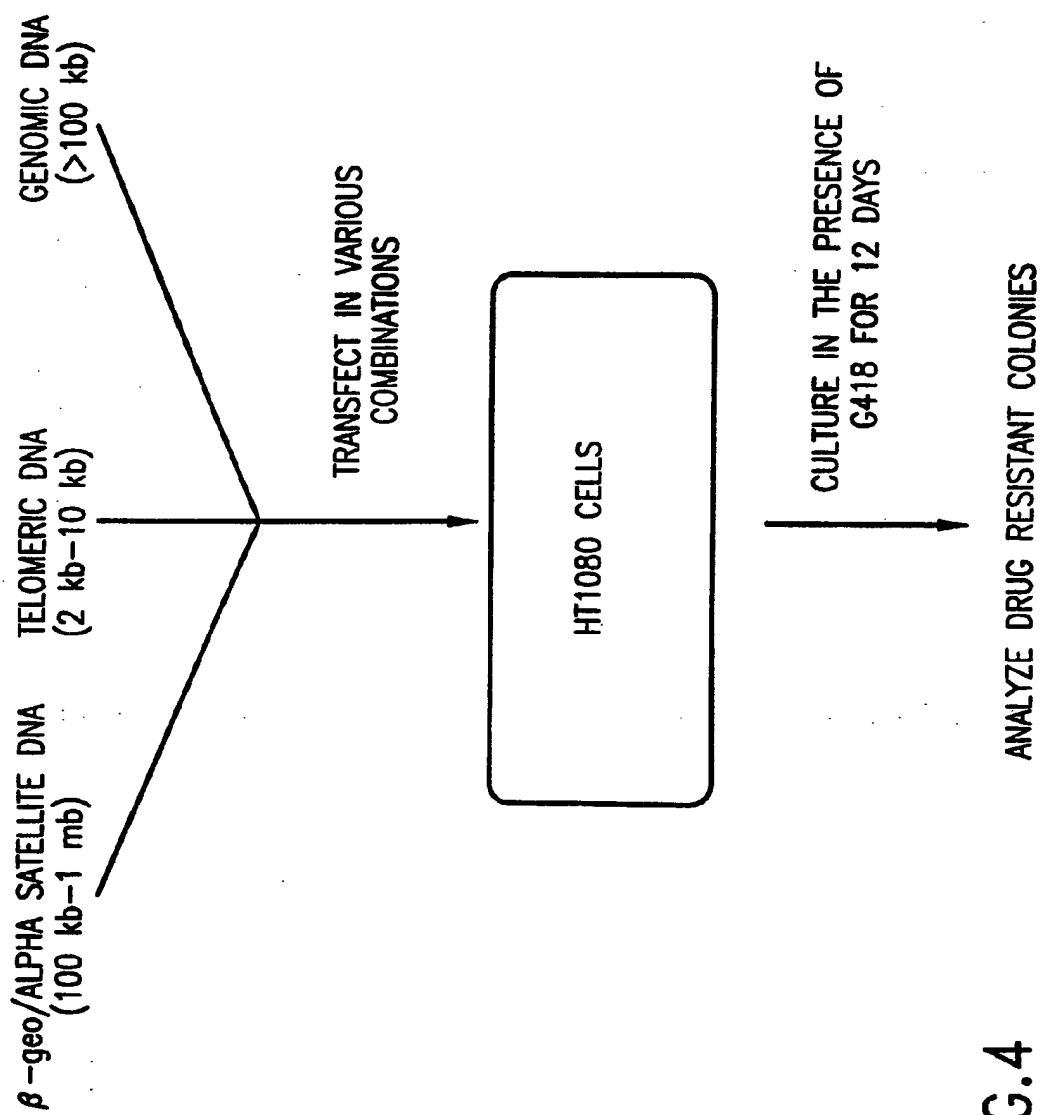


FIG.4

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FIG. 5A



FIG. 5B

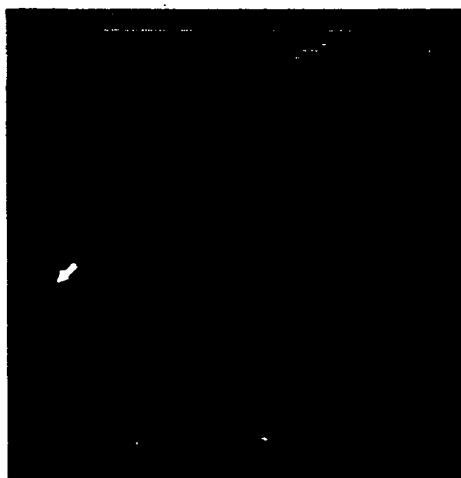


FIG. 5C



FIG. 5D

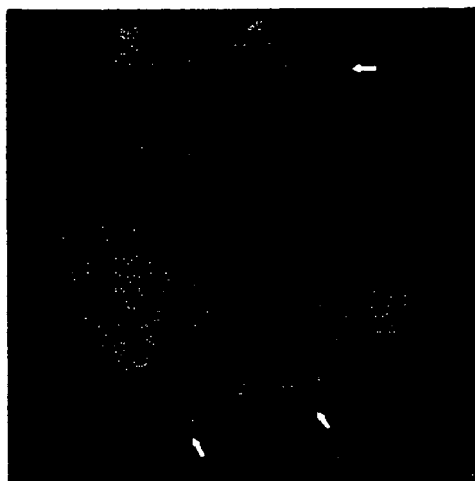


FIG. 6A

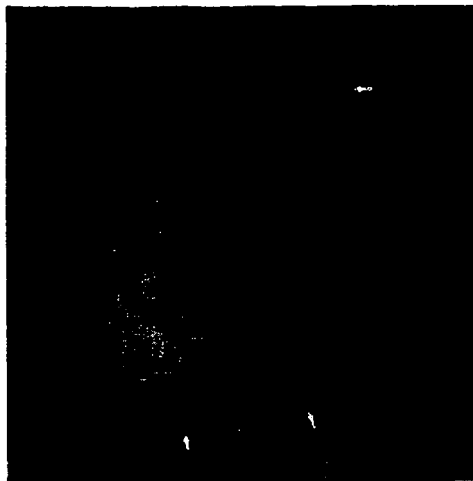


FIG. 6B

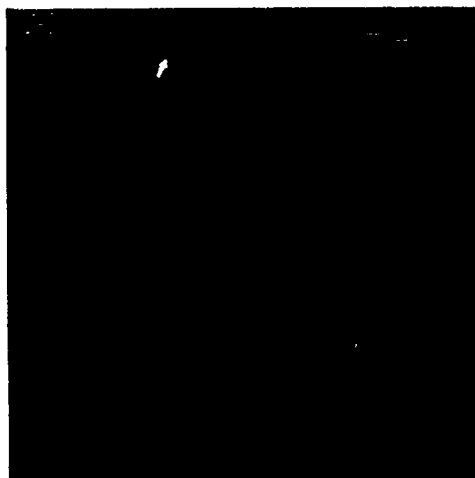


FIG. 6C

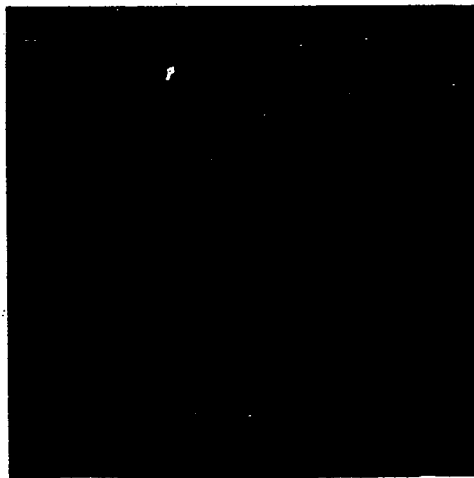


FIG. 6D

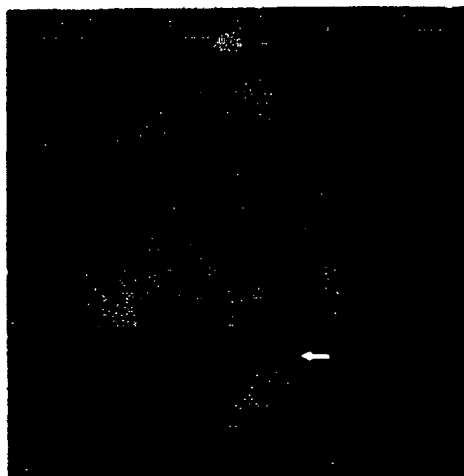


FIG. 7A



FIG. 7B

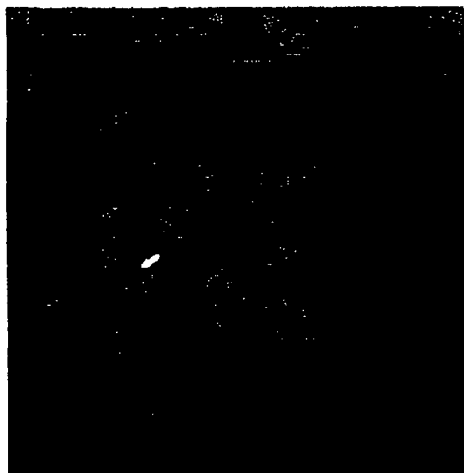


FIG. 7C

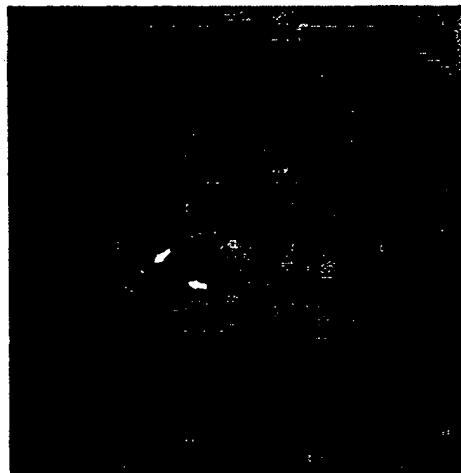


FIG. 7D

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FIG. 8A

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CLONE	TRANSFECTION DNA	ESTIMATED AMOUNT OF SYNTHETIC ALPHA SATELLITE DNA
HT1080	\emptyset	\emptyset
22-6	SYNTHETIC Y ALPHA SATELLITE	450 kb
22-7	SYNTHETIC Y ALPHA SATELLITE	1 mb
22-11	SYNTHETIC Y ALPHA SATELLITE	400 kb
22-13	SYNTHETIC Y ALPHA SATELLITE	2 mb
23-1	SYNTHETIC 17 ALPHA SATELLITE	\emptyset

FIG.8B



FIG. 9A



FIG. 9B

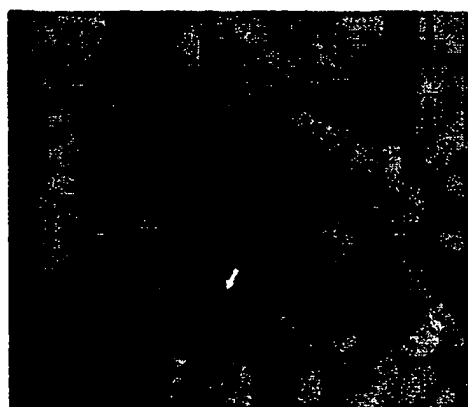


FIG. 9C

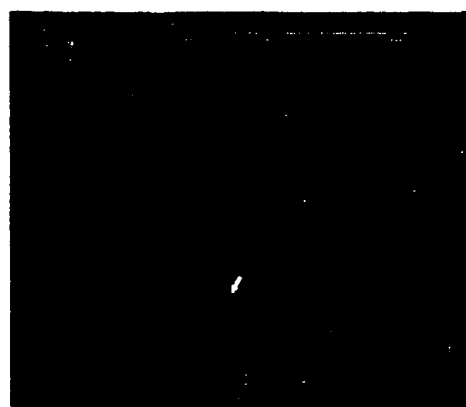


FIG. 9D



FIG. 10B

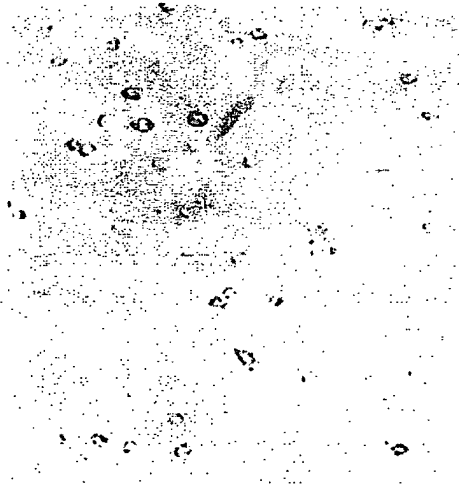


FIG. 10A

INTERNATIONAL SEARCH REPORT

International Application No

PC, /US 96/10248

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 C12N15/90 C12N15/85 C12N15/12 C12N15/62

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 532 050 (BIOLOG RESEARCH CENTER OF THE) 17 March 1993 cited in the application see page 3, line 56 - page 4, line 1; examples 2,3	1,2,18, 35-37, 40-42
X	WO,A,92 07080 (UNIV TEXAS) 30 April 1992 see claims 1,2,8,35	1,2,18, 26, 35-37, 40-42
X	HUMAN MOLECULAR GENETICS, 3 (8). 1994. 1383-1386., XP000601479 TAYLOR S S ET AL: "Addition of functional human telomeres to YACs" see the whole document	1,2,18, 26, 35-37, 40-42
	--- -/-	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
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& document member of the same patent family

Date of the actual completion of the international search

25 September 1996

Date of mailing of the international search report

14. 10. 96

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Authorized officer

Gurdjian, D

INTERNATIONAL SEARCH REPORT

International Application No

PC/US 96/10248

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>GENE THER, JAN 1994, 1 (1) P7-12, ENGLAND, XP000600458</p> <p>HUXLEY C: "Mammalian artificial chromosomes: a new tool for gene therapy." see the whole document</p> <p>---</p>	1-42, 56-68
Y	<p>PROC NATL ACAD SCI U S A, AUG 15 1991, 88 (16) P7006-10, UNITED STATES, XP002014229</p> <p>FARR C ET AL: "Functional reintroduction of human telomeres into mammalian cells." see the whole document</p> <p>---</p>	1-42, 56-68
Y	<p>PROC NATL ACAD SCI U S A, 88 (18). 1991. 8106-8110., XP002014230</p> <p>HADLACZKY G ET AL: "CENTROMERE FORMATION IN MOUSE CELLS COTRANSFORMED WITH HUMAN DNA AND A DOMINANT MARKER GENE" see the whole document</p> <p>---</p>	1-42, 56-68
Y	<p>NUCLEIC ACIDS RES, OCT 11 1995, 23 (19) P3999-4000, ENGLAND, XP002014231</p> <p>KOVACIC RT ET AL: "Protection of megabase DNA from shearing." see the whole document</p> <p>---</p>	8,9,22, 23,30, 31,62,63
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International Application No

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